

08/410,941

DAL

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

IN RE : U.S. Patent No. 5,677,278  
ISSUED: October 14, 1997  
TO: GOSPODAROWICZ, *et al.*  
FOR: TRUNCATED KERATINOCYTE  
GROWTH FACTOR (KGF) HAVING  
INCREASED BIOLOGICAL  
ACTIVITY  
FROM: SERIAL NO. 08/410,941  
FILED: March 27, 1995

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FEB 16 2005

OFFICE OF PETITIONS

Mail Stop Patent Extension  
Commissioner for Patents  
P.O. Box 1450  
Arlington, VA 22313-1450

**REQUEST FOR EXTENSION OF PATENT TERM UNDER 35 U.S.C. §156**

Dear Sirs,

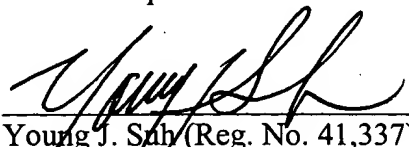
Transmitted herewith are the application papers of Chiron Corporation dated February 8, 2005, for extension of U.S. Patent No. 5,677,278 under 35 U.S.C. §156, based on the regulatory review period for Kevance<sup>TM</sup> (palifermin), together with two duplicate copies as required under 37 C.F.R. § 1.740 (b) and two additional duplicate copies of the application pursuant to M.P.E.P. §2753, for a total of four copies and one original.

As set forth under 37 C.F.R. §1.20(j), please charge the sum of \$1,120.00 to Deposit Account No. 03-1664 for the filing of this application for extension of patent term. Also, please charge any underpayment, or any additional fee that may be required, or credit any overpayment, to Deposit Account No. 03-1664. Two copies of this paper are enclosed.

02/11/2005 HBLAND 00000012 031664 5677278  
01 FC:1457 1120.00 DA

Respectfully submitted,  
Chiron Corporation

Date: Feb. 8, 2005

  
Young J. Suh (Reg. No. 41,337)  
Attorney for Applicant

DAG

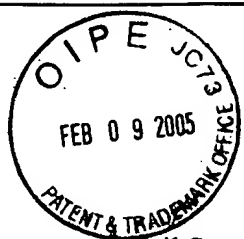
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555 Twelfth Street, NW  
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February 9, 2005



Mail Stop Patent Extension  
Commissioner for Patents  
P.O. Box 1450  
Alexandria, VA 22313-1450

**RECEIVED**

FEB 16 2005

**OFFICE OF PETITIONS**

Re: U.S. Patent No. 5,677,278  
Issued: October 14, 1997  
Title: **Truncated Keratinocyte Growth Factor (KGF) Having  
Increased Biological Activity**  
Applicants: Denis J. GOSPODAROWICZ *et al.*  
Atty. Docket No.: 14483.005

Sir:

The following documents are forwarded herewith for appropriate action by the U.S. Patent and Trademark Office (USPTO):

1. Request for Extension of Patent Term Under 35 U.S.C. §156 (in duplicate);
2. Request for Extension of the Term of United States Patent No. 5,677,278 Under 35 U.S.C. §156 for Kepivance<sup>TM</sup> (Palifermin), including Authorization to Rely Upon Marketing Activities in Request for Extension of Patent Term Under 35 U.S.C. §156; Exhibit A: copy of U.S. Patent No. 5,677,278; Exhibit B: copy of Maintenance Fee Statement; and Exhibit C: copy of Request for Certificate of Correction (together with two duplicate copies as required under 37 C.F.R. § 1.740(b) and two additional duplicate copies pursuant to MPEP § 2753); and
3. Return postcard.

Please stamp the postcard with the filing date of these documents and return it to our courier.

Authorization to charge the \$1,120.00 fee for the filing of this application is given in the accompanying documents.

Respectfully submitted,

*Rachel L. Adams*

David R. Marsh (Reg. No. 41,408)  
Rachel L. Adams (Reg. No. 54,660)

Attachments



IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

IN RE : U.S. Patent No. 5,677,278  
ISSUED: October 14, 1997  
TO: GOSPODAROWICZ, *et al.*  
FOR: TRUNCATED KERATINOCYTE  
GROWTH FACTOR (KGF) HAVING  
INCREASED BIOLOGICAL  
ACTIVITY  
FROM: SERIAL NO. 08/410,941  
FILED: March 27, 1995

Mail Stop Patent Extension  
Commissioner for Patents  
P.O. Box 1450  
Alexandria, VA 22313-1450

RECEIVED  
FEB 16 2005  
OFFICE OF PETITIONS

**REQUEST FOR EXTENSION OF THE TERM OF**  
**UNITED STATES PATENT NO. 5,677,278 UNDER 35 U.S.C. §156**  
**FOR KEPIVANCE™ (PALIFERMIN)**

Dear Sirs,

Chiron Corporation, a corporation organized and existing under the laws of the State of Delaware, and having a place of business at 4560 Horton Street, Emeryville, California, 94662-8097, United States of America, represents that it is the assignee of Letters Patent of the United States No. 5,677,278 granted to Gospodarowicz, *et al.* on October 14, 1997, for Truncated Keratinocyte Growth Factor (KGF) Having Increased Biological Activity, by virtue of assignments, recorded in the United States Patent and Trademark Office (hereinafter referred to as "the Patent Office") on August 12, 1993, at Reel 6638, Frame 0596.

Pursuant to the provisions of 37 C.F.R. §1.730, applicant hereby applies for an extension of the term of Patent No. 5,677,278 under 35 U.S.C. §156 of 1417 days, based on the materials set forth herein and in the accompanying papers.

In the materials which follow herein, numbered paragraphs (1) through (15) correspond to paragraphs (1) through (15) of 37 C.F.R. §1.740(a).

(1) The approved product is Kepivance™ (palifermin). Kepivance™ is a human keratinocyte growth factor (KGF) produced by recombinant DNA technology in *Escherichia coli* (*E coli*). Kepivance™ is a water-soluble, 140 amino acid protein with a molecular weight of 16.3 kilodaltons. It differs from endogenous human KGF in that the first 23 N-terminal amino acids have been deleted to improve protein stability.

**Molecular Weight:**

The molecular weight is 16.3 kilodaltons.

**Structural Formula:**

The amino acid sequence is numbered in accordance with endogenous, full length human KGF. Amino acids 1-54 of endogenous, full length human KGF are absent in Kepivance™ (palifermin). That is, the first 23 amino acids of endogenous, mature human KGF (which are amino acid numbers 32-54 of the endogenous, full length human KGF) are absent in Kepivance™. The CAS registry number for palifermin is 162394-19-6.

	Ser	Tyr	Asp	Tyr	Met	Glu	Gly	Gly	Asp	Ile	
	55					60					
Arg	Val	Arg	Arg	Leu	Phe	Cys	Arg	Thr	Gln	Trp	Tyr
65				70					75		80
Lys	Arg	Gly	Lys	Val	Lys	Gly	Thr	Gln	Glu	Met	Lys
			85					90			95
Ile	Met	Glu	Ile	Arg	Thr	Val	Ala	Val	Gly	Ile	Val
	100					105				110	
Val	Glu	Ser	Glu	Phe	Tyr	Leu	Ala	Met	Asn	Lys	Glu
	115					120				125	
Ala	Lys	Lys	Glu	Cys	Asn	Glu	Asp	Cys	Asn	Phe	Lys
	130				135					140	
Glu	Asn	His	Tyr	Asn	Thr	Tyr	Ala	Ser	Ala	Lys	Trp
145				150					155		160
Gly	Glu	Met	Phe	Val	Ala	Leu	Asn	Gln	Lys	Gly	Ile
			165					170			175
Lys	Lys	Thr	Lys	Lys	Glu	Gln	Lys	Thr	Ala	His	Phe
		180					185				190
Ile	Thr										
	194										

We note that the term “product,” for purposes of patent term extension for a drug product, is defined as “the active ingredient of a new drug, antibiotic drug, or human biological product...including any salt or ester of the active ingredient, as a single entity or in combination with another active ingredient.” 35 U.S.C. § 156(f)(2). Nothing in this application should be construed as limiting the term product for purposes of the requested patent term extension to the specific form of human keratinocyte growth factor approved in Kepivance™.

(2) Kepivance™ (palifermin) was subject to regulatory review under Section 351 of the Public Health Service Act, 42 U.S.C. § 262.

(3) Kepivance™ (palifermin) received permission for commercial marketing or use under section Section 351 of the Public Health Service Act, 42 U.S.C. § 262, on December 15, 2004.

(4) The active ingredient in Kepivance™ (palifermin) is a human keratinocyte growth factor (KGF) produced by recombinant DNA technology in *Escherichia coli* (*E coli*). That active ingredient has not been previously approved for commercial marketing or use under the Federal Food, Drug and Cosmetic Act, the Public Health Service Act or the Virus-Serum-Toxin Act.

(5) This application is being submitted within the sixty day period permitted for its submission pursuant to 37 C.F.R. § 1.720(f). The last day on which this application could be submitted is February 11, 2005.

(6) The patent for which an extension is being sought is identified as follows:

Inventors: Dennis J. Gospodarowicz and Frank R. Masiarz  
Patent No.: 5,677,278  
For: TRUNCATED KERATINOCYTE GROWTH FACTOR (KGF)  
HAVING INCREASED BIOLOGICAL ACTIVITY  
Issued: October 14, 1997  
Expires: October 14, 2014

(7) A copy of U.S. Patent No. 5,677,278, the patent for which an extension is being sought, is attached hereto as EXHIBIT A.

(8) One maintenance fee payment for U.S. Patent No. 5,677,278 was made to keep the patent in force beyond four years from its issue date (a copy of the receipt of such payment is included herewith as EXHIBIT B). On January 11, 2005, Applicants filed a Request for Certificate of Correction of Office Mistake for U.S. Patent No. 5,677,278 (a copy of the Request for Certificate of Correction is included herewith as EXHIBIT C). Pursuant to 37 C.F.R. § 1.740(a)(8), Applicants will submit as a supplement to this Request for Extension of Patent Term Under 35 U.S.C. § 156 a copy of any Certificate of Correction, if granted, for U.S. Patent No. 5,677,278.

(9) Patent No. 5,677,278 claims the approved product. Claims 1-6 and 10 claim the approved product.

Claim 1 of Patent No. 5,677,278 reads on Kepivance™ because:

Kepivance™ is a keratinocyte growth factor fragment that exhibits at least a 2-fold increase in mitogenic activity as compared to a mature, recombinant, full-length keratinocyte growth factor, wherein the fragment lacks the first 23 N-terminal amino acid residues of the mature, full-length keratinocyte growth factor but retains the remainder of the molecule.

(10) The relevant dates and information pursuant to 35 U.S.C. §156(g) in order to enable the Secretary of Health and Human Services to determine the applicable regulatory review period are as follows:

- An exemption under subsection (i) of section 505 of the Federal Food, Drug and Cosmetic Act became effective for Kepivance™ (palifermin) on December 2, 1995, following receipt by the Food and Drug Administration of an Investigational New Drug (“IND”) Application, identified by FDA as BB-IND 6370, on November 2, 1995.
- A Biologics License Application (“BLA”) under section 351 of the Public Health Service Act for Kepivance™ (palifermin) was completed as follows:
  - Reviewable Unit 1 of the BLA was submitted to FDA on May 14, 2004<sup>1</sup>
  - Reviewable Unit 2 of the BLA was submitted on June 15, 2004.
  - This BLA was assigned the number 125103/0
- BLA No. 125103/0 was approved on December 15, 2004.

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<sup>1</sup> Because this application was reviewed by FDA as part of its “Fast Track” program, the BLA was submitted in two stages. We believe that the first submission date constitutes the date that this application “was initially submitted” for purposes of 35 U.S.C. § 156(g). Accordingly, we have calculated the proposed period of extension on that basis. However, if it is determined that the date of the second submission is the appropriate one to be used for this purpose, 16 days should be subtracted from the period of extension we have calculated.

(11) A brief description of the significant activities undertaken by the marketing applicant during the applicable regulatory review period with respect to the approved product and the significant dates applicable to such activities follows:

The investigational new drug application ("IND") for this drug was submitted on November 1, 1995. It was received by FDA on November 2 and became effective on December 2, 1995. Amgen conducted a series of clinical investigations of this drug. On December 31, 2003, FDA granted fast track designation to this product. The first reviewable unit of the biologic license application ("BLA") for this product was submitted on May 14, 2004, with a subsequent submission of the second reviewable unit on June 17, 2004. The BLA was approved on December 15, 2004.

The following chart identifies significant events and communications of substance with the FDA concerning this product:

<u>Date</u>	<u>Regulatory Event</u>
Nov. 1, 1995	Submission of IND
Nov. 7, 1995	FDA letter acknowledging receipt of IND on Nov. 2, 1995 and assigning identifier BB-IND 6370 to IND
Dec. 2, 1995	Effective Date of IND
Dec. 13, 1995	Submission of study protocols 950224 & 950225
Jan. 31, 1996	Submission of study protocols 950275 & 950226
May 6, 1996	Submission of proposed revised clinical development plan
Jul. 17, 1997	Submission of study protocol 960136
Jul. 31, 1997	Submission of study protocol 970147
Sept. 19, 1997	Submission of study protocol 960189
Dec. 10, 1997	Submission of study protocol 970189
Mar. 19, 1998	Submission of study protocol 970149
Dec. 22, 1998	Submission of study protocol 980231
Sept. 30, 1999	Meeting with FDA to discuss clinical development of KGF
Dec. 17, 1999	Teleconference with FDA to discuss clinical development issues



Sept. 5, 2000	End of Phase 3 Meeting with FDA
Aug. 6, 2001	Type C Meeting with FDA
Sept. 7, 2001	Teleconference with FDA to discuss proposed quality of life trial
Nov. 16, 2001	FDA letter explaining clinical hold on proposed study
Dec. 26, 2001	Submission of response to clinical hold
Mar. 28, 2002	FDA letter explaining clinical hold on proposed study
Apr. 24, 2002	Submission of response to clinical hold
May 10, 2002	FDA letter lifting partial clinical hold
Aug. 23, 2002	Submission of study protocol 20010192
Oct. 30, 2002	Submission of study protocols 20010182 & 990123
Sept. 21, 2003	pre-BLA Meeting with FDA
Dec. 2, 2003	CMC Meeting with FDA
Dec. 5, 2003	Submission of request for fast track designation
Dec. 31, 2003	FDA letter granting fast track designation
May 14, 2004	Submission of initial components of BLA
Jun. 10, 2004	Meeting with FDA Review Division
Jun. 15, 2004	Submission of additional components of BLA
Jul. 9, 2004	Submission of immunogenicity data
Jul. 20, 2004	Submission of revised immunogenicity data
Aug. 13, 2004	FDA notification that the Kepivance BLA is accepted for filing
Aug. 27, 2004	FDA letter containing questions regarding immunogenicity data and inspectional readiness at the drug substance manufacturing facility
Oct. 15, 2004	Teleconference with FDA concerning review schedule

Nov. 15, 2004     FDA discipline review letter for module 4 the BLA received by Amgen

Dec. 15, 2004     Amgen receives FDA approval letter

(12) In the opinion of the Applicant, U.S. Patent No. 5,677,278 is eligible for an extension under 35 U.S.C. §156. The length of extension claimed is 1417 days.

The length of extension of term of Patent No. 5,677,278 of 1417 days claimed by applicant was determined according to the provisions of 37 C.F.R. §1.775 as follows:

- According to 37 C.F.R. §1.775(b), the length of extension is equal to the regulatory review period for the approved product, reduced as appropriate pursuant to paragraphs (d)(1) through (d)(6) of 37 C.F.R. §1.775.
- According to 37 C.F.R. §1.775(c), the regulatory review period is the sum of: (A) the number of days in the period beginning on the date the exemption under subsection 505 of the Federal Food, Drug and Cosmetic Act became effective for the approved product and ending on the date the BLA was initially submitted under Section 351 of the Public Health Service Act; and (B) the number of days in the period beginning on the date the BLA was initially submitted under Section 351 of the Public Health Service Act and ending on the date the BLA was approved. The exemption under subsection 505(i) of the Federal Food, Drug and Cosmetic Act became effective on Dec. 2, 1995; and the BLA was approved on December 15, 2004. Hence the regulatory review period under 37 C.F.R. §1.775(c) is the sum of the period from December 2, 1995 to May 14, 2004 and from May 14, 2004 to December 15, 2004. This is the sum of 3301 days.
- According to 37 C.F.R. §1.775(d)(1)(i), the number of days in the regulatory review period which were on and before the date on which the patent issued must be subtracted. Patent No. 5,677,278 issued on October 14, 1997. Subtraction of the period on or before October 14, 1997 leaves a reduced regulatory review period from October 15, 1997 to May 14, 2004 and from May 14, 2004 to December 15, 2004. This is the sum of 2403 days and 215 days, which is 2618 days.
- 37 C.F.R. §1.775(d)(1)(ii) does not apply.
- According to 37 C.F.R. §1.775 (d)(1)(iii), the regulatory review period must then be reduced by one-half of the days remaining in the period defined in 37 C.F.R. §1.775(c)(1). This is one-half of 2403 days, which, disregarding half days, is 1201 days. After subtraction, this now leaves a reduced regulatory review period of 1202 days plus 215 days, which is 1417 days.
- According to 37 C.F.R. §1.775(d)(2), the reduced regulatory review period of 1417 days must be added to the expiration date of Patent No. 5,677,278, i.e., October 14, 2014. This gives a date of August 31, 2018.
- According to 37 C.F.R. §1.775(d)(3), 14 years must be added to the date of approval of the approved product. This gives a date of December 15, 2018.
- According to 37 C.F.R. §1.775(d)(4), the earlier of these dates must be selected. The earlier date of these dates is August 31, 2018.
- The provisions of 37 C.F.R. §1.775(d)(5) apply to this application because Patent No. 5,677,278 issued after September 24, 1984. Pursuant to 37 C.F.R. §1.775(d)(5)(i), five (5) years are added to the expiration date of Patent No. 5,677,278 (October 14, 2014) giving a date of October 14, 2019. According to 37 C.F.R. §1.775(d)(5)(ii), the dates obtained pursuant to 37 C.F.R.

§1.775(d)(5)(i) and 37 C.F.R. §1.775(d)(4) are compared and the earlier date is selected. The date calculated according to 37 C.F.R. §1.775(d)(4) above is August 31, 2018. Therefore, the earlier of these dates is August 31, 2018. Applicant is entitled to an extension of term of Patent No. 5,677,278 until August 31, 2018, i.e., an extension of 1417 days from original expiration date of October 14, 2014.

- The provisions of 37 C.F.R. §1.775(d)(6) do not apply because Patent No. 5,677,278 issued on October 14, 1997, after September 24, 1984.

(13) Applicant acknowledges a duty to disclose to the Director of United States Patent and Trademark Office and the Secretary of Health and Human Services any information which is material to the determination of entitlement to the extension which is being sought to the term of Patent No. 5,677,278.

(14) The prescribed fee under 37 C.F.R. §1.20(j) for receiving and acting on this application for patent term extension is to be charged to Deposit Account No. 03-1664.

(15) Please direct all inquiries and correspondence relating to this application for patent term extensions as follows:

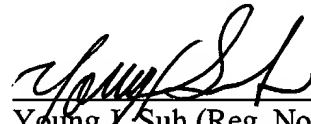
David R. Marsh  
Arnold & Porter LLP  
555 Twelfth Street, N.W.  
Washington, D.C. 20004-1206  
(202) 942-5068 telephone  
(202) 942-5999 facsimile

Pursuant to 37 C.F.R. §1.740(b), two duplicate copies of these application papers are enclosed herewith. Pursuant to M.P.E.P. §2753 an additional two copies of the application are also enclosed herewith. Accordingly, a total of one original application for patent term extension of Patent No. 5,677,278 and four copies of the application are submitted herewith.

Applicant respectfully requests prompt and favorable action on the merits of this application for extension of the term of Letters Patent No. 5,677,278 of 1417 days, based on the regulatory review period for Kepivance™ (palifermin).<sup>2</sup>

Respectfully submitted,  
Chiron Corporation

Date: Feb. 8, 2005

  
\_\_\_\_\_  
Young J. Suh (Reg. No. 41,337)  
Attorney for Applicant

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<sup>2</sup> As noted in footnote 1, *supra*, this total would be reduced by 16 days, to 1401 days, if the PTO were to conclude that this application was not "initially submitted" for purposes of 35 U.S.C. § 156(g) until the second reviewable unit of that BLA was submitted on June 15, 2004.



THE UNITED STATES PATENT AND TRADEMARK OFFICE

IN RE : U.S. Patent No. 5,677,278  
ISSUED: October 14, 1997  
TO: GOSPODAROWICZ, *et al.*  
FOR: TRUNCATED KERATINOCYTE  
GROWTH FACTOR (KGF) HAVING  
INCREASED BIOLOGICAL  
ACTIVITY  
FROM: SERIAL NO. 08/410,941  
FILED: March 27, 1995

Mail Stop Patent Extension  
Commissioner for Patents  
P.O. Box 1450  
Arlington, VA 22313-1450

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**AUTHORIZATION TO RELY UPON MARKETING ACTIVITIES**  
**IN REQUEST FOR EXTENSION OF PATENT TERM UNDER 35 U.S.C. §156**

Dear Sirs,

Pursuant to M.P.E.P. § 2752, Amgen Inc., a corporation organized and existing under the laws of the State of Delaware, and having a place of business at One Amgen Center Drive, Thousand Oaks, California, 91320, United States of America, hereby authorizes Chiron Corporation to rely upon the activities of Amgen Inc. before the Food and Drug Administration during the regulatory review period for Kepivance™ (palifermin) in its request for extension of patent term under 35 U.S.C. § 156 of U.S. Patent No. 5,677,278, granted to Gospodarowicz, *et al.* on October 14, 1997, and assigned to Chiron Corporation by virtue of assignments recorded in the United States

Patent and Trademark Office on August 12, 1993, at Reel 6638, Frame 0596. Amgen Inc., effective December 23, 2002, is the licensee of Chiron Corporation with respect to U.S. Patent No. 5,677,278.

Respectfully submitted,  
Amgen Inc.

Date: 2/7/05

RTT

Robert Ramos (Reg. No. 37,915)  
Attorney for Marketing Applicant

## Exhibit A





US005677278A

**United States Patent** [19]  
**Gospodarowicz et al.**

[11] **Patent Number:** **5,677,278**  
 [45] **Date of Patent:** **Oct. 14, 1997**

[54] **TRUNCATED KERATINOCYTE GROWTH  
 FACTOR (KGF) HAVING INCREASED  
 BIOLOGICAL ACTIVITY**

[75] **Inventors:** Denis J. Gospodarowicz, Lafayette;  
 Frank R. Masiarz, San Francisco, both  
 of Calif.

[73] **Assignee:** Chiron Corporation, Emeryville, Calif.

[21] **Appl. No.:** 410,941

[22] **Filed:** Mar. 27, 1995

#### **Related U.S. Application Data**

[63] **Continuation of Ser. No. 86,427, Jun. 29, 1993, abandoned.**

[51] **Int. Cl.<sup>6</sup>** ..... A61K 38/18; C07K 14/475

[52] **U.S. Cl.** ..... 514/12; 514/2; 514/8;  
 530/350; 530/397; 530/399

[58] **Field of Search** ..... 435/69.1, 320.1,  
 435/69.4; 530/399, 350, 397, 324, 325,  
 326, 327, 328, 329, 330; 536/23.51; 514/12,  
 863

[56] **References Cited**

#### **FOREIGN PATENT DOCUMENTS**

WO 90/08771 8/1990 WIPO.

#### **OTHER PUBLICATIONS**

Bowie et al. 1990. Science 247: 1306-1310.

Bellosta, P. et al., J. Cell Biol. (1993) 121(3):705-713.

Finch, P.W. et al., Science (1989) 2:752-755.

Ron, D. et al., J. Biol. Chem. (1993) 268(4):2984-2988.

*Primary Examiner*—Stephen G. Walsh

*Assistant Examiner*—Elizabeth C. Kemmerer

*Attorney, Agent, or Firm*—Paul B. Simboli; Grant D. Green;  
 Robert P. Blackburn

[57]

#### **ABSTRACT**

The present invention relates to a keratinocyte growth factor fragment, KGF<sub>des1-23</sub>, or an analog thereof that is composed of a portion of an amino acid sequence of mature, full length keratinocyte growth factor, KGF<sub>163</sub>. The fragment exhibits at least a 2-fold increase in mitogenic activity as compared to a mature, recombinant keratinocyte growth factor, rKGF, but lacks a sequence comprising the first 23 amino acid residues, C-N-D-M-T-P-E-Q-M-A-T-N-V-N-C-S-S-P-E-R-H-T-R- (SEQ ID NO: 2) of the KGF<sub>163</sub> N-terminus. The present invention also relates to a DNA molecule encoding KGF<sub>des1-23</sub>, an expression vector and a transformed host containing the DNA molecule, and a method of producing KGF<sub>des1-23</sub> by culturing the transformed host. The present invention further relates to a conjugate of KGF<sub>des1-23</sub> and a toxin molecule, and the use thereof for treatment of hyperproliferative disease of the epidermis. Moreover, the present invention relates to a therapeutic composition containing KGF<sub>des1-23</sub> and a pharmaceutically acceptable carrier and the use thereof for wound healing purposes.

**13 Claims, 5 Drawing Sheets**



FIG. 1

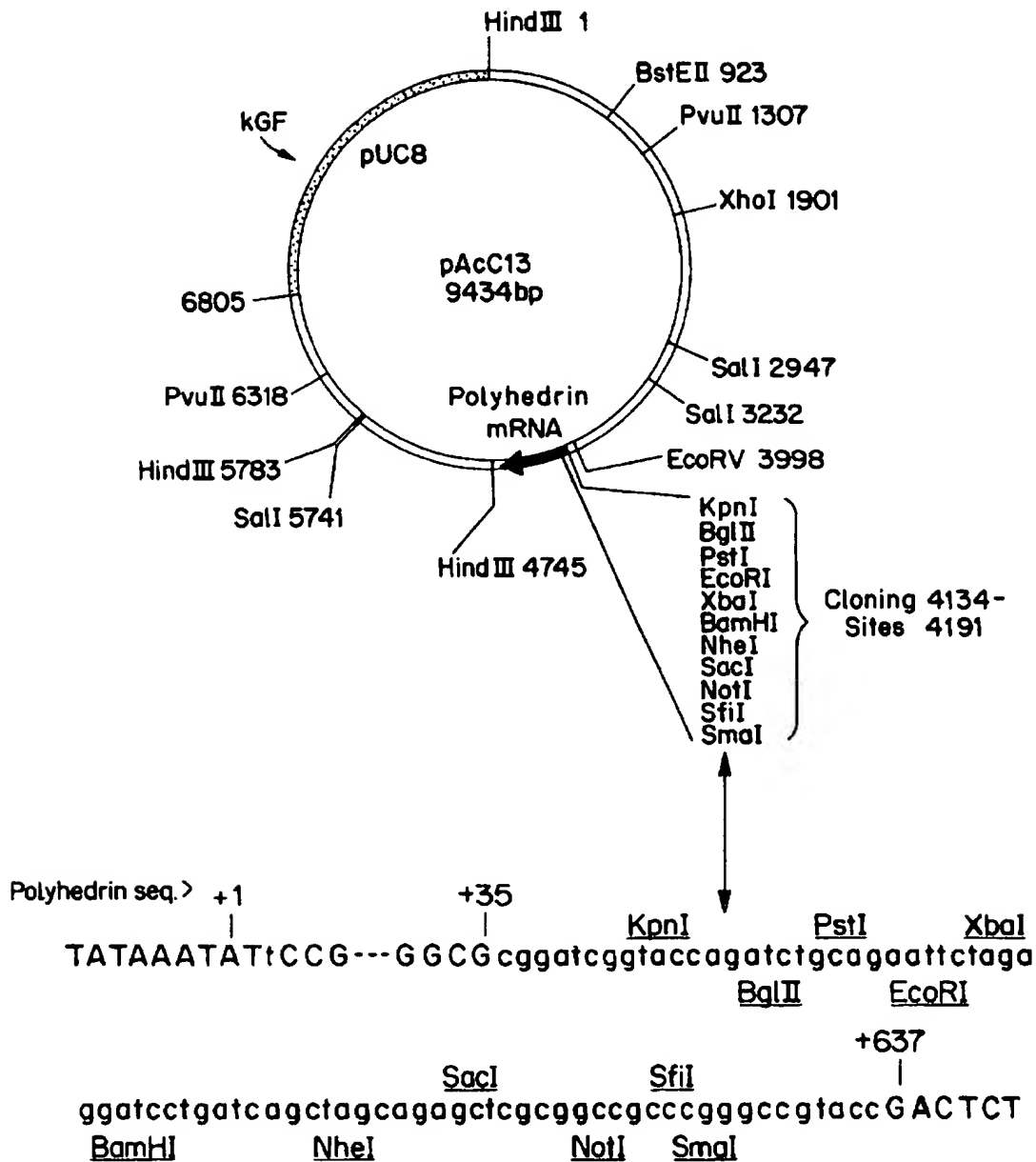


FIG. 2

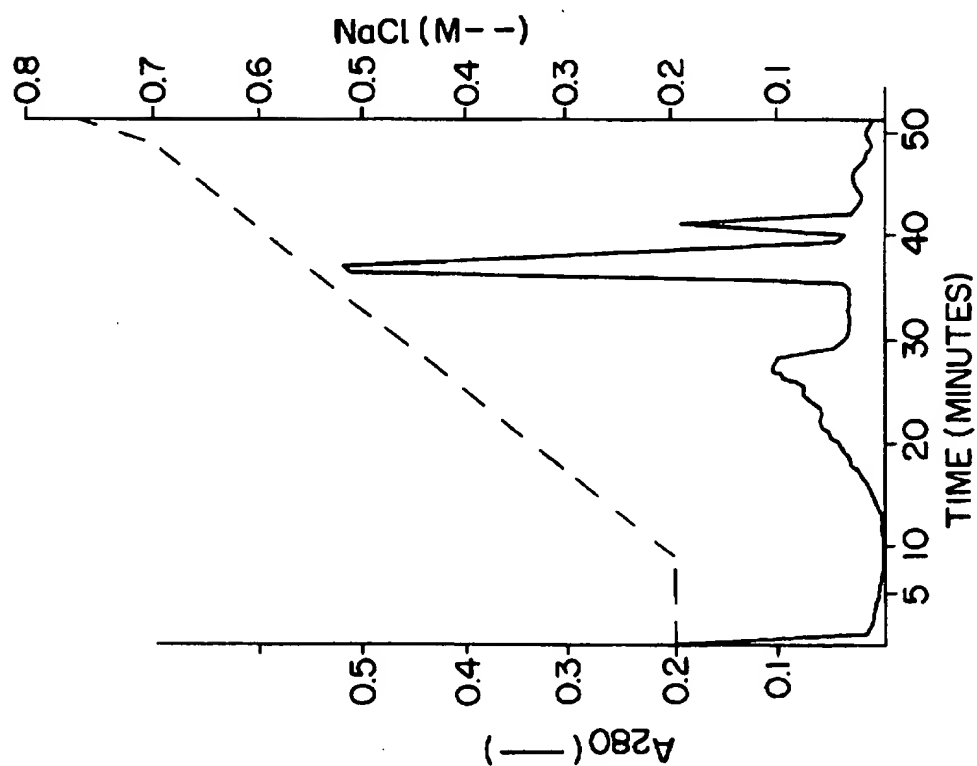


FIG. 3A

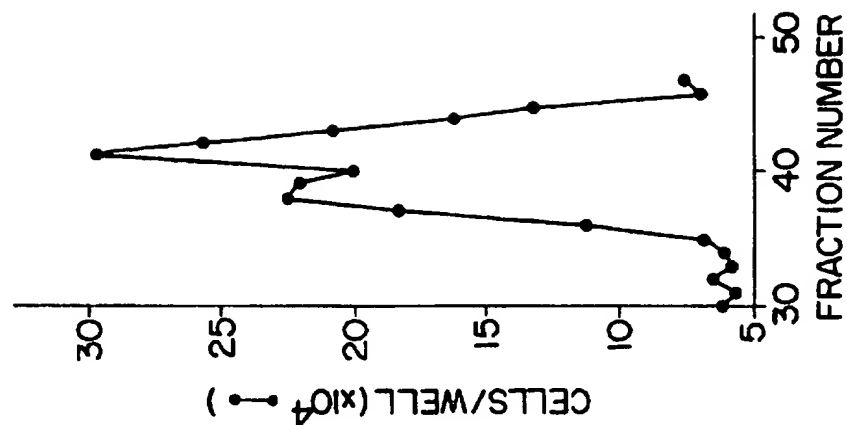


FIG. 3B

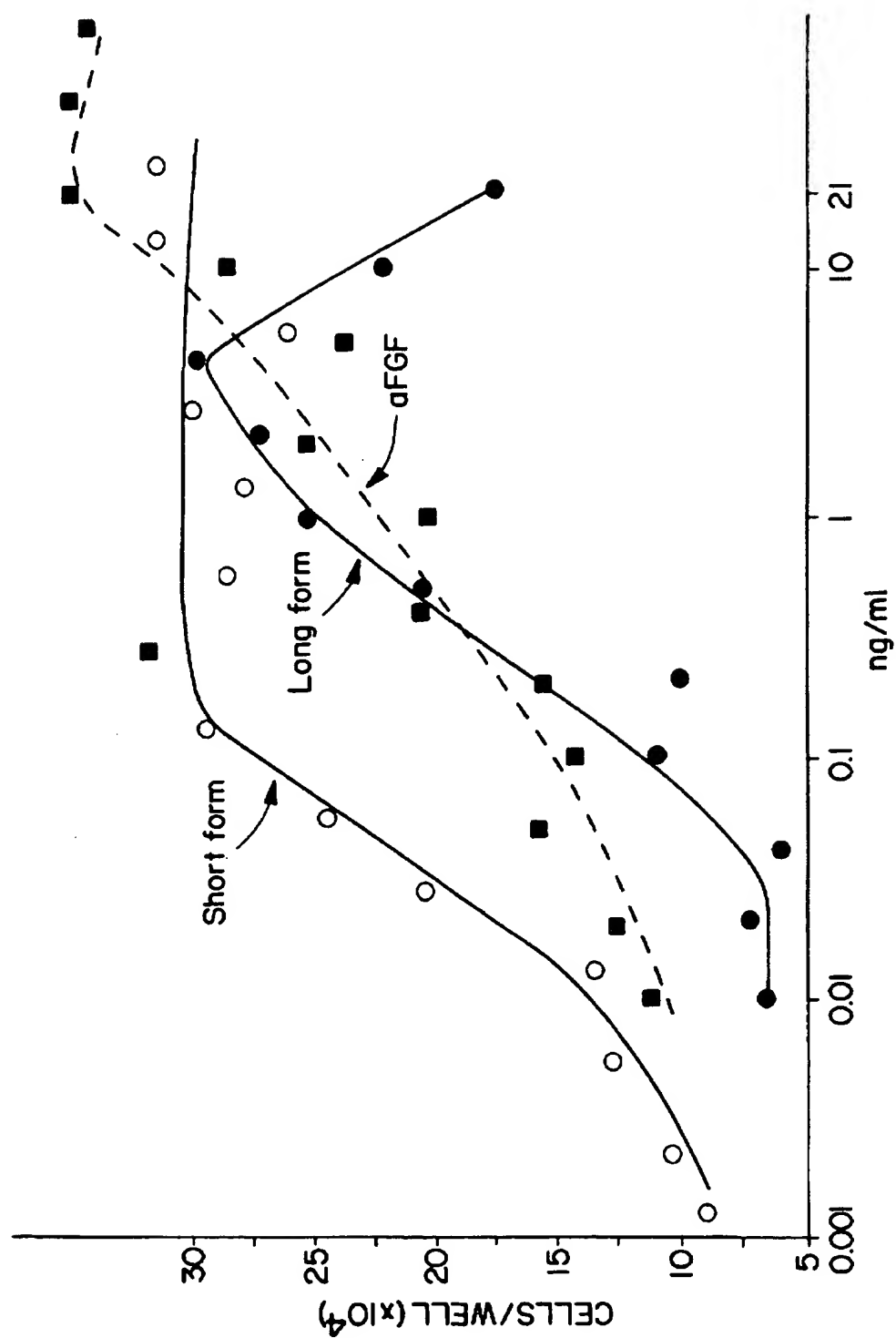


FIG. 4

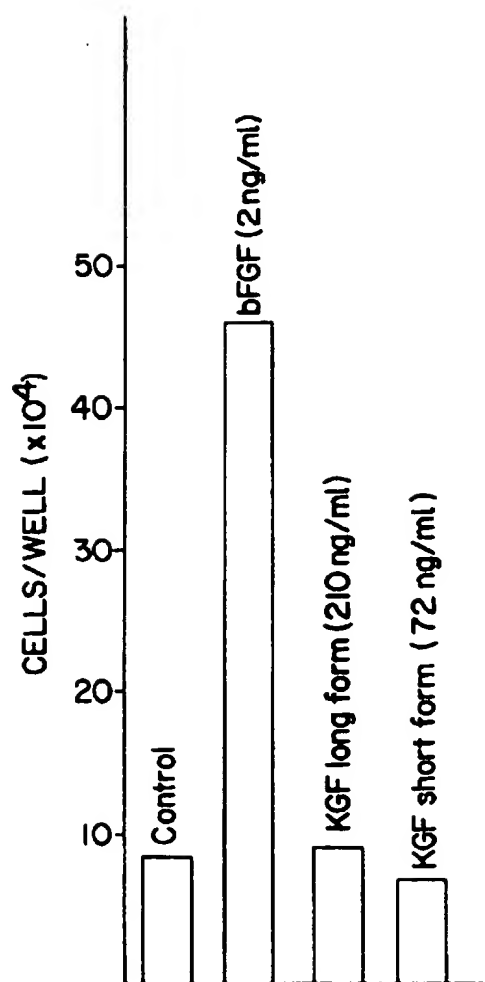


FIG. 5A

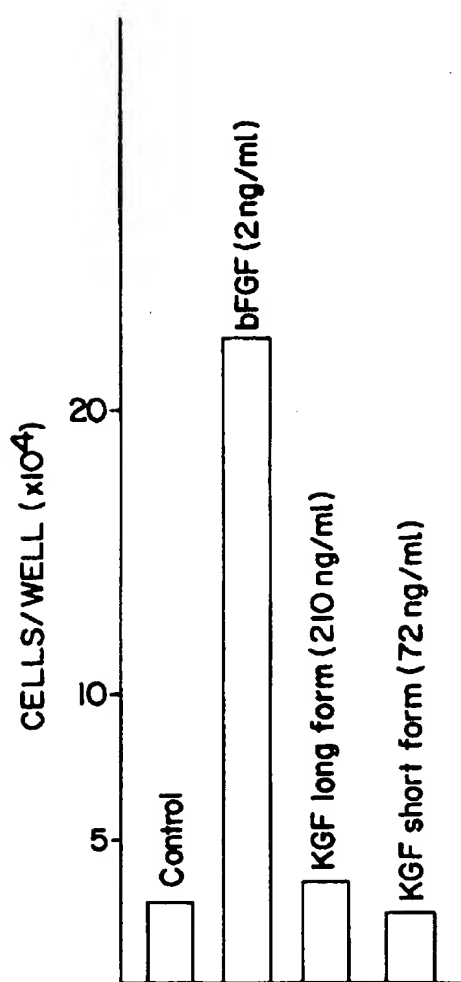


FIG. 5B

# TRUNCATED KERATINOCYTE GROWTH FACTOR (KGF) HAVING INCREASED BIOLOGICAL ACTIVITY

This application is a continuation of application Ser. No. 08/086,427 filed on 29 Jun. 1993, now abandoned.

## FIELD OF THE INVENTION

The present invention relates generally to keratinocyte growth factor ("KGF"). More specifically, this invention relates to a KGF fragment and analogs thereof having increased biological activity, and decreased cytotoxicity as compared to a mature, recombinant, full length KGF expressed in an insect cell expression system. This KGF fragment lacks the first 23 amino acid residues of the N-terminus of mature, full-length KGF, including a potential glycosylation site. The N-terminus was previously believed to confer upon KGF its epithelial cell specificity.

KGF belongs to the family of fibroblast growth factors ("FGFs"), the prototype of which is represented by basic FGF ("bFGF"). KGF is, hence, also known as FGF-7. Like other FGFs, KGF is a heparin-binding protein, but unlike other FGFs, it has a unique target cell specificity. In particular, FGFs are generally capable of stimulating the proliferation and differentiation of a variety of cell types derived from the primary or secondary mesoderm as well as from neuroectoderm. KGF is similar to other FGFs in its ability to stimulate epithelial cell proliferation, but is dissimilar to other FGFs in its inability to stimulate endothelial cells or fibroblast proliferation, as discussed in Finch, P. W. et al., *Science* 245: 752-755 (1989). Mature, full-length KGF, designated herein as KGF<sub>163</sub>, is a polypeptide with 163 amino acid residues, and possesses a potential N-glycosylation site at amino acid 14 of the consensus sequence for glycosylation that extends from amino acid residue 14 to 16 at the N-terminus, as indicated in Finch et al. (1989), loc. cit.

## BACKGROUND OF THE INVENTION

FGFs, including acidic fibroblast growth factor ("aFGF") and basic fibroblast growth factor ("bFGF"), are known to have heparin-binding properties and have the ability to induce the differentiation and proliferation of ventral, as well as dorsal, mesoderm in early blastulae, as discussed in Gospodarowicz et al., *Cell. Biol. Rev.* 25:307-314 (1991), and Basilico et al., *Adv. Cancer Res.* 59:115-165 (1992). The response of cells to FGF is mediated through binding thereof to cell surface receptors known as fibroblast growth factor receptors ("FGFRs"), of which there are three inter-related types, as discussed in Hou et al., *Science* 251:665-668 (1991). High affinity FGFRs are tyrosine kinases and include the flg receptor ("FGFR-1"), the bek receptor ("FGFR-2"), and the K Sam receptor ("FGFR-3"), as discussed in Lee et al., *Science* 245:57-60 (1989); Dionne et al., *EMBO J.* 9:2685-2692 (1990); Miki et al., *Science* 251:72-75 (1991); Miki et al., *Proc. Natl. Acad. Sci. USA* 89:246-250 (1992); and Dell et al., *J. Biol. Chem.* 267:21225-21229 (1992).

Both FGFR-1 and FGFR-2 are widely expressed in mesodermal and neuroectodermal tissues, and both are able to bind aFGF and bFGF with similar affinities. FGFR-3, also referred to as KGFR, is a KGF receptor that is specific to epithelial cells. It is an alternative transcript of FGFR-2. In contrast to FGFR-2, which shows high affinity for both aFGF and bFGF and no affinity for KGF, FGFR-3 binds KGF and aFGF with an affinity approximately 20 to 1000

fold higher than bFGF, as discussed in Miki et al. (1992), and Dell et al. (1992), loc. cit.

The tightly restricted tissue distribution of KGFR to epithelial cells and, therefore, the tissue restricted activity of KGF, is desirable in many types of wound healing applications, as well as in the treatment of hyperproliferative diseases of the epidermis, such as psoriasis and basal cell carcinoma. Presently, except for KGF, no highly suitable factor exists for these applications. It would be desirable, therefore, if KGF could be modified to increase its potency and decrease its cytotoxicity for therapeutic applications.

Recently, Ron et al., *J. Biol. Chem.* 268:2984-2988 (February 1993) found that when KGF<sub>163</sub> was expressed in a prokaryotic expression system, a recombinant KGF ("rKGF") polypeptide could be obtained that possessed mitogenic activity. When the rKGF molecule was truncated by deletion of 3, 8, 27, 38, and 48 amino acid residues from the N-terminus of the mature KGF<sub>163</sub> polypeptide, biological activity of the resulting molecules varied. With deletion of 3 and 8 amino acid residues, respectively, the mitogenic activity of the resulting molecules did not appear to be affected as compared to full-length rKGF. Deletion of 27 amino acid residues, however, resulted in molecules that display 10-20 fold reduced mitogenic activity. Deletion of 38 and 48 amino acid residues, respectively, resulted in complete loss of mitogenic activity and heparin-binding ability. Ron et al., however, failed to produce any truncated rKGF fragments that possessed increased mitogenic activity as compared to the full-length rKGF molecule.

## SUMMARY OF THE INVENTION

One of the objects of the present invention is to provide a KGF fragment or an analog thereof that contains a portion of the amino acid sequence of mature, full-length KGF and that exhibits at least a 2-fold, but preferably 7-fold, more preferably, a 7-10 fold increase in mitogenic activity as compared to the mature, full-length rKGF. This KGF fragment lacks a sequence comprising the first 23 N-terminal amino acid residues, C-N-D-M-T-P-E-Q-M-A-T-N-V-N-C-S-S-P-E-R-H-T-R- (SEQ ID NO: 2), of the mature, full-length rKGF.

Another one of the objects of the present invention is to provide a KGF fragment, as above, that has decreased cytotoxicity as compared to the mature, full-length rKGF.

Still another one of the objects of the present invention is to provide a conjugate that comprises the KGF fragment described above and a toxin molecule. The toxin molecule can be one of a ricin A molecule, a diphtheria toxin molecule, or a saporin molecule.

Yet another one of the objects of the present invention is to provide a therapeutic composition that contains the KGF fragment as described above and a pharmaceutically acceptable carrier, for example, one suitable for topical application to human skin.

Still another one of the objects of the present invention is to provide a DNA molecule that is composed of a nucleotide sequence that encodes the KGF fragment described above.

Yet another one of the objects of the present invention is to provide an expression vector that contains the DNA molecule that encodes the KGF fragment above and a regulatory sequence for expression of the DNA molecule. The expression vector can be, for example, a yeast, a bacterial, a mammalian or a baculovirus expression vector.

Yet another one of the objects of the present invention is to provide a host cell transformed with the expression vector

described above. The host cell can be, for example, a prokaryote such as a bacterial cell, or a eukaryote such as a yeast cell, a mammalian cell, or an insect cell.

Yet another one of the objects of the present invention is to provide a method of producing the KGF fragment by culturing the transformed host cell as described above and isolating the KGF fragment from the culture.

Still another one of the objects of the present invention is to provide a method of stimulating epithelial cell growth by applying the KGF fragment to an area in which epithelial cell growth is desired and allowing the cells to grow.

Still another one of the objects of the present invention is to provide a method for wound healing by applying the therapeutic composition described above to an area of a wound to be treated and allowing the wound to heal.

Still another one of the objects of the present invention is to provide a method of treating a hyperproliferative disease of the epidermis by applying the conjugate described above to an area to be treated.

Further objects, features, and advantages of the present invention would be apparent to a person of ordinary skill in the art and need not be enumerated here.

#### SUMMARY OF THE DRAWINGS

FIG. 1 shows the amino acid sequence of the mature, full-length, human KGF polypeptide, beginning with the N-terminus and containing 163 amino acid residues, having a single consensus sequence for glycosylation at amino acid residues 14-16. The amino acid sequence of the mature KGF polypeptide corresponds to amino acids nos. 32 to 194 of SEQ ID NO: 1.

FIG. 2 shows the pAcC13 expression vector into which the 163 amino acid sequence of KGF has been inserted. The polylinker and flanking sequences shown in FIG. 2 are referred as SEQ ID NO: 19 in the Sequence Listing.

FIG. 3 illustrates the protein elution profile (A) and bioactivity profile (B) of the rKGF obtained from a Mono S HR5/5 cation exchange FPLC column.

FIG. 4 compares the biological activity of the long, i.e., full-length rKGF, and the short, i.e., rKGF<sub>des1-23</sub>, form of KGF versus aFGF on the cells of the Balb/Mk cell line. The figure legend for FIG. 4 is as follows: ○=KGF<sub>des1-23</sub>, ●=KGF<sub>163</sub>, and □=aFGF.

FIG. 5 compares the biological activity of the long, i.e., full-length rKGF, and the short, i.e., rKGF<sub>des1-23</sub>, form of KGF versus bFGF on vascular endothelial cells derived from large vessels (A:ABAE cells) or capillary cells (B:ACE cells).

#### DETAILED DESCRIPTION OF THE PREFERRED EMBODIMENTS

It has been surprisingly discovered that a truncated, unglycosylated KGF having a deletion spanning the first 23 N-terminal amino acid residues of mature recombinant KGF ("rKGF"), herein designated the KGF fragment or KGF<sub>des1-23</sub>, possesses a much greater biological activity and decreased cytotoxicity on epithelial cells as compared with the mature, full-length rKGF. Generally, the KGF fragment of the present invention retains the specificity of KGF for stimulation of epithelial cell proliferation.

The following definitions are incorporated herein and are provided for a better understanding of the present invention.

#### Definitions

As used herein, the term "keratinocyte growth factor" or "KGF" refers to a member of a group of structurally distinct

proteins known as FGFs that display varying degrees of sequence homology, suggesting that they are encoded by a related family of genes. The FGFs share common receptor sites on cell surfaces. KGF, for example, can bind to FGFR-3.

"Mature, full-length KGF" or "long form of KGF" or "KGF<sub>163</sub>," as used herein refers to the mature polypeptide that contains 163 amino acid residues, as shown in FIG. 1, and represented by amino acid residues 32 to 194 in SEQ ID NO: 1.

As used herein, "the KGF fragment" or "short form of KGF" or "KGF<sub>des1-23</sub>" refers to a polypeptide that is a truncated version of KGF<sub>163</sub>, lacking the first 23 amino acid residues at the N-terminus of KGF<sub>163</sub>. The sequence of the 23 deleted amino acid residues is shown in SEQ ID NO: 2. The properties of the KGF fragment include (i) its biological activity such as at least a 2-fold, preferably, a 7-fold and, more preferably, a 7-10 fold increase in stimulation of epithelial cell proliferation as compared to the rKGF<sub>163</sub> molecule, and (ii) its ability to bind to FGFR-3. The biological activity of the KGF fragment can be measured, for example, by the Balb/Mk cell proliferation assay, described in Example 5, section C.

An "analog of KGF<sub>163</sub>" or "analog of the KGF fragment" herein refers to amino acid insertions, deletions, or substitutions in the relevant molecule that do not substantially affect its properties. The KGF<sub>des1-23</sub> analog herein retains at least the 2-fold increase, preferably, the 7-fold increase, more preferably, the 7-10 fold increase in mitogenic activity as compared to that of rKGF<sub>163</sub>. For example, the analog herein can include conservative amino acid substitutions in the rKGF<sub>des1-23</sub> molecule.

"Conservative amino acid substitutions" herein are, for example, those that take place within a family of amino acids that are related in their side chains. The families of amino acids include (1) acidic: aspartic acid, glutamic acid; (2) basic: lysine, arginine, histidine; (3) non-polar: alanine, valine, leucine, isoleucine, proline, phenylalanine, methionine, tryptophan; and (4) uncharged polar: glycine, asparagine, glutamine, cysteine, serine, threonine, tyrosine. Phenylalanine, tryptophan, and tyrosine are sometimes classified jointly as a family of aromatic amino acids. In particular, it is generally accepted that an isolated replacement of a leucine with an isoleucine or valine, or an aspartic acid with a glutamic acid, or a threonine with a serine, or a similar conservative substitutions of an amino acid with a structurally related amino acid, in an area outside of the polypeptide's active site will not have a major effect on the properties of the polypeptide.

The term "recombinant" as used herein in relation to a polynucleotide intends a polynucleotide of semisynthetic, or synthetic origin, or encoded by cDNA or genomic DNA ("gDNA") such that (1) it is not associated with all or a portion of a polynucleotide with which it is associated in nature, (2) is linked to a polynucleotide other than that to which it is linked in nature, or (3) does not occur in nature.

A "expression vector" is a polynucleotide that is operable in a desired host cell and capable of causing the production of the KGF fragment. Examples of expression vectors are plasmids, integrating vectors, etc.

A "regulatory sequence" refers to a polynucleotide sequence that is necessary for regulation of expression of a coding sequence to which the polynucleotide sequence is operably linked. The nature of such regulatory sequences differs depending upon the host organism. In prokaryotes, such regulatory sequences generally include, for example, a



promoter, and/or a transcription termination sequence. In eukaryotes, generally, such regulatory sequences include, for example, a promoter and/or a transcription termination sequence. The term "regulatory sequence" may also include additional components the presence of which are advantageous, for example, a secretory leader sequence for secretion of the polypeptide attached thereto.

"Operably linked" refers to a juxtaposition wherein the components so described are in a relationship permitting them to function in their intended manner. A regulatory sequence is "operably linked" to a coding sequence when it is joined in such a way that expression of the coding sequence is achieved under conditions compatible with the regulatory sequence.

A "coding sequence" is a polynucleotide sequence that is translated into a polypeptide, usually via mRNA, when placed under the control of an appropriate regulatory sequence. The boundaries of the coding sequence are generally determined by a translation start codon at its 5'-terminus and a translation stop codon at its 3'-terminus. A coding sequence can include, but is not limited to, cDNA, and recombinant polynucleotide sequences.

An "open reading frame" (ORF) is a region of a polynucleotide sequence that encodes a polypeptide. This region may encode a precursor form of a mature polypeptide or just the polypeptide.

"PCR" refers to the techniques of the polymerase chain reaction as described in Saiki, et al., *Nature* 324:163 (1986); and Scharf et al., *Science* 233: 1076-1078 (1986); U.S. Pat. No. 4,683,195; and U.S. Pat. No. 4,683,202. As used herein, x is "heterologous" with respect to y if x is not naturally associated with y or x is not associated with y in the same manner as is found in nature.

"Homology" refers to the degree of sequence identity between x and y. Typically, the sequence identity between x and y will be at least 50%, usually, the sequence identity will be no less than 60%; more typically, the sequence identity will be no less than 75%; preferably no less than 80%; and even more preferably at least 90%. Most preferably, the sequence identity between x and y will be at least 95%, even more preferably at least 98%, even more preferably at least 99%.

As used herein, the term "polypeptide" refers to a polymer of amino acids and does not refer to a specific length of the product. Thus, peptides, oligopeptides, and proteins are included within the definition of polypeptide. This term also does not exclude post-expression modifications of the polypeptide, for example, glycosylations, acetylations, phosphorylations and the like. Included within the definition are, for example, polypeptides containing one or more analogs of an amino acid (including, for example, unnatural amino acids), polypeptides with substituted linkages, as well as other modifications known in the art, both naturally occurring and non-naturally occurring.

As used herein, "terminators" are regulatory sequences, such as polyadenylation and transcription termination sequences, located 3' or downstream of the stop codon of the coding sequences.

"Recombinant host cells," "host cells," "cells," "cell cultures," and other such terms denote, for example, microorganisms, insect cells, and mammalian cells, that can be or have been used as recipients for introduction of recombinant vector or other transfer DNA, and include the progeny of the cell that has been transformed. Such progeny includes those that may not necessarily be identical in morphology or in genomic or total DNA complement as the

original parent that may be produced as a result of natural, accidental, or deliberate mutation. Examples of mammalian host cells include Chinese hamster ovary ("CHO") and monkey kidney ("COS") cells.

As used herein, the term "microorganism" includes prokaryotic and eukaryotic microbial species such as bacteria and fungi, the latter including yeast and filamentous fungi.

"Transformation," as used herein, refers to the transfer of an exogenous polynucleotide into a host cell, irrespective of the method used for the transfer, which can be, for example, by infection, direct uptake, transduction, F-mating, microinjection or electroporation. The exogenous polynucleotide may be maintained as a non-integrated vector, for example, a plasmid, or alternatively, may be integrated into the host genome.

"Purified" and "isolated" in reference to a polypeptide or a nucleotide sequence means that the indicated molecule is present in substantial absence of other biological macromolecules of the same species or type. The term "purified" as used herein means at least 75% by weight; preferably, at least 85% by weight, more preferably, at least 95% by weight and, most preferably, at least 98% by weight, of biological macromolecules of the same type are present, but water, buffers, and other small molecules, especially molecules having a molecular weight of less than 1000, can be present as well.

By "pharmaceutically acceptable carrier," is meant any carrier that is used by persons in the art for administration into a human that does not itself induce any undesirable side effects such as the production of antibodies, fever, etc. Suitable carriers are typically large, slowly metabolized macromolecules that can be a protein, a polysaccharide, a polylactic acid, a polyglycolic acid, a polymeric amino acid, amino acid copolymers or an inactive virus particle. Such carriers are well known to those of ordinary skill in the art. Preferably the carrier is thyroglobulin.

A "therapeutic composition" herein contains one or more pharmaceutically acceptable carriers, and one or more additional component such as water, saline, glycerol, or ethanol. Additionally, auxiliary substances, such as wetting or emulsifying agents, pH buffering substances, and the like, may be present in such compositions.

By "a therapeutically effective amount," as used herein refers to that amount that is effective for production of a desired result. This amount varies depending upon the health and physical condition of the individual to be treated, the capacity of the individual's immune system to synthesize antibodies, the degree of protection desired, the formulation, the attending physician's assessment of the medical situation, and other relevant factors. It is expected that the amount will fall in a relatively broad range that can be determined through routine trials.

Accordingly, a preferred embodiment of the present invention is a novel unglycosylated KGF fragment, KGF<sub>der1-23</sub>, unaccompanied by impurities which normally accompany the native molecule when it is produced in vivo. This fragment has an apparent molecular weight of about 18 kilo-Daltons ("kD") based upon its migration in Sodium Dodecyl Sulfate Polyacrylamide Gel Electrophoresis ("SDS/PAGE") as a single band (results not shown). The specific activity of purified KGF<sub>der1-23</sub> on Balb/Mk cells is measured by its ED<sub>50</sub> value, as defined by the concentration that causes half maximal stimulation of cell proliferation. The ED<sub>50</sub> of the KGF fragment herein is found to average about 40 pg/ml. The bioactivity of the KGF fragment herein

will be at least about 2-fold, preferably, about 7-fold and, more preferably, about 7-10 fold greater than that of the full-length rKGF protein or that of aFGF, when compared in a cell proliferation assay, and 100-fold greater than when rKGF<sub>163</sub> is bioassayed, using initiation of DNA synthesis in Balb/Mk cells maintained in a chemically defined medium, as described in PCT Patent Application, No. WO 90/08771.

In a preferred embodiment of the present invention, KGF<sub>der1-23</sub> is produced by recombinant DNA technology, particularly in the case of large-scale commercial production. A recombinant DNA molecule and an expression vector comprising KGF<sub>der1-23</sub> in accordance with the present invention can be made and expressed by conventional gene expression technology using methods well-known in the art, as discussed in more detail below.

The present invention also includes analogs of the rKGF fragment that retain the epithelial cell specificity and the at least 2-fold increase in mitogenic activity of rKGF<sub>der1-23</sub>, as compared to the activity of rKGF<sub>163</sub>. In a preferred embodiment, the analog retains the 7-fold, more preferably, the 7-10 fold and, most preferably, the 10-fold increase in mitogenic activity of rKGF<sub>der1-23</sub>. Analogs of rKGF<sub>der1-23</sub> include post-translationally modified versions of rKGF<sub>der1-23</sub>, for example, those generated by glycosylations, acetylations, or phosphorylations thereof. Analogs of rKGF<sub>der1-23</sub> can be also made by conventional techniques of amino acid substitution, deletion, or addition, for example, by site-directed mutagenesis. Thus, all references to embodiments of the present invention as it relates to rKGF<sub>1-23</sub> apply equally to analogs thereof.

In one embodiment of the present invention, the KGF fragment can be made by isolating native, mature KGF from cells producing the same and deleting the first 23 N-terminal amino acid residues therefrom. Such deletion can be performed by any conventional techniques known in the art.

In an alternative embodiment, the KGF fragment can be made by isolating the coding sequence of native KGF<sub>163</sub>, deleting the codons that encode the first 23 N-terminal amino acid residues, inserting the modified coding sequence into an expression vector, and transforming host cells with the expression vector to produce the recombinant KGF<sub>der1-23</sub>.

In a further embodiment of the present invention, the KGF fragment can be made by isolating the coding sequence of KGF<sub>163</sub> from cells known to produce KGF, inserting the coding sequence of KGF<sub>163</sub> into a baculovirus expression vector, transforming a host insect cell with the baculovirus expression vector, and harvesting and isolating the approximately 18 kD molecular species from the transformed insect cell culture that possesses increased KGF activity, using conventional separatory techniques.

In another embodiment of the present invention, an expression vector containing the KGF<sub>der1-23</sub> coding sequence can be produced by operably linking the KGF<sub>der1-23</sub> to one or more regulatory sequences such that the resulting vector is operable in a desired host.

In a further embodiment of the present invention, the coding sequence of KGF<sub>der1-23</sub> can be obtained by conventional techniques, including the isolation of the coding sequence of KGF<sub>163</sub> from a cDNA library known to contain such, and deleting therefrom the sequence encoding the first 23 N-terminal amino acid residues. Deletion of the coding sequence of the N-terminal amino acids can be accomplished in vivo or in vitro. The former can be achieved, for example, by expression of the KGF<sub>163</sub> coding sequence in a baculovirus/insect cell expression system. The latter can be

achieved by known PCR techniques using primers that exclude the N-terminal sequences.

In a further embodiment of the present invention, the DNA or vector comprising the coding sequence of KGF<sub>der1-23</sub> can be expressed in a prokaryotic or eukaryotic expression system, in particular, a bacterial, mammalian, yeast, or insect cell expression system. In a preferred embodiment, a bacterial or yeast cell expression system may be ideal for production of the KGF<sub>der1-23</sub> fragment. The yeast cell can be, for example, *Saccharomyces cerevisiae*.

In another embodiment of the present invention, the KGF fragment can be expressed as a fusion protein by linking, in the correct frame and orientation, the 5' end of the KGF<sub>der1-23</sub> coding sequence to the coding sequence of another molecule that facilitates either intracellular or extracellular production of the rKGF<sub>der1-23</sub>. The coding sequence of such other molecules can be, for example, at least a portion of the prepro  $\alpha$ -factor leader sequence, for extracellular expression; the superoxide dismutase ("SOD") gene sequence, or the ubiquitin gene sequence, for intracellular expression in yeast cells.

In yet another embodiment of the present invention, the rKGF<sub>der1-23</sub> polypeptide can be conjugated to other molecules suitable for its intended use. For example, the KGF<sub>der1-23</sub> polypeptide can be conjugated to a toxin molecule, such as ricin A, diphtheria toxin, or saporin for destruction of its target cell, i.e., epithelial cells, particularly, keratinocytes.

In a further embodiment, the KGF<sub>der1-23</sub> polypeptide or a conjugate thereof can be mixed with a pharmaceutically acceptable carrier to produce a therapeutic composition that can be administered for therapeutic purposes, for example, for wound healing, and for treatment of hyperproliferative diseases of the skin and tumors, such as psoriasis and basal cell carcinoma.

The KGF fragment of the present invention can be used for identification of receptor recognition sites as well as for the design of peptide agonists or antagonists. Moreover, in view of the unique specificity of KGF for keratinocytes, its inability to induce the proliferation of vascular endothelial cells or fibroblasts, and its lack of cytotoxicity, KGF<sub>der1-23</sub> should be a preferred agent of choice for wound healing applications, particularly where there is a desire to promote re-epithelialization of the skin. KGF<sub>der1-23</sub> should also be particularly useful in corneal epithelial repair. Other applications of KGF<sub>1-23</sub> utilize its specificity for epithelial cells found in the gastrointestinal tract.

The choice to select the KGF fragment herein over other growth factors such as epidermal growth factor ("EGF"), platelet-derived growth factor ("PDGF"), and other FGFs for skin repair is within the skill of a person in the art. These other growth factors, for example, induce fibroplasia and angiogenesis, in addition to stimulating, either directly or indirectly, keratinocyte proliferation. In skin repair, such additional activities could produce undesirable side effects such as scarring. In corneal repair involving either a wound or surgery, the use of these factors could induce blood vessel invasion into the cornea, and result in corneal opacity or edema. KGF, on the other hand, has a unique specificity for keratinocytes and does not induce the proliferation of vascular endothelial cells or fibroblasts and, therefore, would be the agent of choice for these particular wound healing applications.

The practice of the present invention will employ, unless otherwise indicated, conventional techniques of molecular biology, microbiology, recombinant DNA, and immunology,

which are within the skill of the art. Such techniques are explained fully in the literature, including Sambrook, et al., *MOLECULAR CLONING: A LABORATORY MANUAL* 2nd ed. (Cold Spring Harbor Laboratory Press, 1989); DNA CLONING, Vol. I and II, D. N. Glover ed. (IRL Press, 1985); OLIGONUCLEOTIDE SYNTHESIS, M. J. Gait ed. (IRL Press, 1984); NUCLEIC ACID HYBRIDIZATION, B. D. Hames & S. J. Higgins eds. (IRL Press, 1984); TRANSCRIPTION AND TRANSLATION, B. D. Hames & S. J. Higgins eds., (IRL Press, 1984); ANIMAL CELL CULTURE, R. I. Freshney ed. (IRL Press, 1986); IMMOMOBILIZED CELLS AND ENZYMES, K. Mosbach (IRL Press, 1986); B. Perbal, *A PRACTICAL GUIDE TO MOLECULAR CLONING*, Wiley (1984); the series, *METHODS IN ENZYMOLOGY*, Academic Press, Inc.; GENE TRANSFER VECTORS FOR MAMMALIAN CELLS, J. H. Miller and M. P. Calos eds. (Cold Spring Harbor Laboratory, 1987); *METHODS IN ENZYMOLOGY*, Vol. 154 and 155, Wu and Grossman, eds., and Wu, ed., respectively (Academic Press, 1987); IMMUNOCHEMICAL METHODS IN CELL AND MOLECULAR BIOLOGY, R. J. Mayer and J. H. Walker, eds. (Academic Press London, Harcourt Brace U.S., 1987); PROTEIN PURIFICATION: PRINCIPLES AND PRACTICE, 2nd ed. (Springer-Verlag, N.Y. (1987), and HANDBOOK OF EXPERIMENTAL IMMUNOLOGY, Vol. I-IV, D. M. Weir et al., (Blackwell Scientific Publications, 1986); Kitts et al., *Biotechniques* 14:810-817 (1993); Munemitsu et al., *Mol. and Cell. Biol.* 10:5977-5982 (1990).

Standard abbreviations for nucleotides and amino acids are used in FIG. 1 and elsewhere in this specification. All publications, patents, and patent applications cited herein are incorporated by reference.

It has been unexpectedly found that when KGF vector is expressed in insect cells, *Spodoptera frugiperda* ("SF9") by infection of a recombinant baculovirus, *Autographa californica*, containing the cDNA coding for the mature form of KGF (163 amino acids), they were capable of producing a KGF fragment, a truncated form of KGF that lacks the first 23 amino acid residues of the native KGF N-terminal domain that contains a single glycosylation site. This truncated and unglycosylated KGF fragment designated herein as KGF<sub>des1-23</sub> or KGF<sub>140</sub>, in contrast to the native long form identified as KGF<sub>163</sub>, has a 7- to 10-fold increased potency on target cells. The target cell specificity of KGF<sub>des1-23</sub> is unchanged. Additionally, at high concentrations of the KGF fragment, no toxic effect on keratinocytes is observed in contrast to that observed for KGF<sub>163</sub>. These observations suggest that, contrary to what was previously proposed in PCT Application, Publication No. WO 90/08771, the target cell specificity of KGF does not reside in its N-terminal domain. Furthermore, the present invention shows that an N-terminally truncated version of KGF in fact represents an improved KGF version with higher biological activity and decreased cytotoxicity for therapeutic application.

Further, KGF<sub>des1-23</sub> exhibits a pI more basic than that for KGF<sub>163</sub>. In one assay, the pI was approximately 9.9 for KGF<sub>des1-23</sub> and 9.4 for KGF<sub>des1-23</sub>. KGF<sub>des1-23</sub> also exhibits a higher affinity for heparin and for the Mono S resin.

Recombinant KGF<sub>des1-23</sub>, in accordance with the present invention, can be made by well-known recombinant techniques. In this regard, the KGF<sub>des1-23</sub> coding sequence is operably linked to one or more regulatory sequences in the suitable vector in a proper reading frame and orientation. The vector is suitable when it can replicate or can be

replicated to express the recombinant protein in a particular host. Thus, the coding sequence of KGF<sub>des1-23</sub> can be inserted, for example, into a yeast expression vector for expression in yeast cells, a bacterial expression vector for expression in bacterial cells, a mammalian vector for expression in mammalian cells.

In a preferred embodiment, the coding sequence of KGF<sub>des1-23</sub> for expression purposes herein is a complementary DNA ("cDNA") molecule encoding KGF<sub>des1-23</sub>. The KGF<sub>des1-23</sub> cDNA can be made by known recombinant techniques, such as isolating total cellular RNA from a host cell known to express KGF, isolating poly A<sup>+</sup> RNA by running the total cellular RNA through an oligo-dT column and eluting the poly A<sup>+</sup> RNA therefrom, constructing a cDNA library, using reverse transcriptase, based on the poly A<sup>+</sup> RNA isolated, which contains mRNA, and selecting the cDNA clones that contain the KGF<sub>des1-23</sub> coding sequence by use of labeled oligonucleotide probes constructed on the basis of the known amino acid sequence of KGF<sub>des1-23</sub>.

A regulatory sequence that can be linked to the KGF<sub>des1-23</sub> coding sequence in the expression vector herein is a promoter that is operable in the host cell in which the recombinant KGF<sub>des1-23</sub> is to be expressed. Optionally, other regulatory sequences can be used herein, such as one or more of an enhancer sequence, an intron with functional splice donor and acceptance sites, a signal sequence for directing secretion of the recombinant KGF<sub>des1-23</sub>, a polyadenylation sequence, other transcription terminator sequences, and a sequence homologous to the host cell genome. Other sequences, such as an origin of replication, can be added to the vector as well to optimize expression of the desired product. Further, a selectable marker can be present in the expression vector for selection of the presence thereof in the transformed host cells.

The regulatory sequences can be derived from various sources. For example, one or more of them can be associated with a native KGF coding sequence, or derived from or homologous with the host cell in which the sequence is to be expressed, or derived from a microbial, for example, bacterial or yeast source, or hybrids thereof. For use herein, the KGF<sub>des1-23</sub> coding sequence is, preferably, operably linked downstream of the promoter sequence and upstream of the terminator sequence.

The various components of the expression vector can be linked together directly or, preferably, via linkers that constitute sites of recognition by restriction enzymes. In one embodiment of the present invention, a promoter sequence herein is linked directly with the KGF<sub>des1-23</sub> coding sequence, in which case the first amino acid at the N-terminal of the recombinant KGF<sub>des1-23</sub> protein would be methionine, which is encoded by the ATG start codon. The methionine residue at the N-terminal can be optionally cleaved from the recombinant protein by conventional techniques, for example, in vitro incubation with cyanogen bromide provided no other methionine residues are present in the fragment.

Any promoter that would allow expression of the KGF fragment in a desired host can be used in the present invention. Yeast promoter sequences that are associated with polynucleotide sequences encoding enzymes in the fermentative metabolic pathway are particularly useful in the present invention. Examples of these enzymes include alcohol dehydrogenase ("ADH"), as described in European Patent Application, Publication No. 284 044, enolase, glucokinase, glucose-6-phosphate isomerase, glyceraldehyde-3-phosphate-dehydrogenase ("GAP" or

"GAPDH"), hexokinase, phosphofructokinase, 3-phosphoglycerate mutase, and pyruvate kinase ("PyK"), as described in European Patent Application, Publication No. 329 203. In addition, the yeast PHO5 gene, encoding acid phosphatase, as described in Myanohara et al., *Proc. Natl. Acad. Sci. USA* 80:1 (1983), is also useful as a promoter sequence herein.

Other yeast promoters that are suitable herein include, for example, those described in Cohen et al., *Proc. Natl. Acad. Sci. USA* 77:1078 (1980); Henikoff et al., *Nature* 283:835 (1981); Hollenberg et al., *Curr. Topics Microbiol. Immunol.* 96:119 (1981); Hollenberg et al., "The Expression of Bacterial Antibiotic Resistance Genes in the Yeast *Saccharomyces cerevisiae*," in *PLASMIDS OF MEDICAL, ENVIRONMENTAL AND COMMERCIAL IMPORTANCE*, K. N. Timmis and A. Puhler, eds. (Amsterdam/New York 1979); Mercerau-Puigalon et al., *Gene* 11:163 (1980); Panthier et al., *Curr. Genet.* 2:109 (1980).

Prokaryotic promoter sequences, optionally containing operator portions, that can be used herein include  $\beta$ -lactamase (penicillinase) and lactose promoter systems, as described in Chang et al., *Nature* 198:1056 (1977), tryptophan promoter system, as described in Goeddel et al., *Nucleic Acids Res.* 8:4057 (1980), and the  $\lambda$  (lambda)-derived  $P_L$  promoter.

Preferred mammalian promoter sequences that can be used herein are those from mammalian viruses that are highly expressed and that have a broad host range. Examples include the SV40 early promoter, the Cytomegalovirus ("CMV") immediate early promoter mouse mammary tumor virus long terminal repeat ("LTR") promoter, adenovirus major late promoter (Ad MLP), and Herpes Simplex Virus ("HSV") promoter. In addition, promoter sequences derived from non-viral genes, such as the murine metallothionein gene, are also useful herein. These promoters can further be either constitutive or regulated, such as those that can be induced with glucocorticoids in hormone-responsive cells.

Promoters that can be used for insect cell expression in the present invention include the baculovirus polyhedron hybrid promoter and the p10 promoter.

Further, promoters for use in the present invention can be synthetic hybrid promoters that do not occur in nature ("non-natural promoters"), but contain a regulatory region linked with a heterologous expression initiation region. For example, the UAS sequence of one yeast promoter may be joined with the transcription activation region of another yeast promoter, creating a synthetic hybrid promoter. Examples of such promoters include the ADH regulatory sequence linked to the GAP transcription activation region, as described in U.S. Pat. Nos. 4,876,197 and 4,880,734. Other examples include those containing regulatory sequences that are associated with the coding sequences encoding ADH2, GAL4, GAL10, or PHO5, combined with the transcriptional activation region of a glycolytic enzyme, such as GAP or PyK, as described in U.S. Pat. Nos.: 4,876,197 and 4,880,734.

Bacterial hybrid promoters include, for example, the tac promoter, as described in De Boer et al. (1983), that is derived from sequences of the trp and lac UV5 promoters. A functional non-natural promoter for use herein may also be a synthetic promoter that is based on a consensus sequence of different promoters.

In another embodiment of the present invention, an enhancer element can be combined with a promoter sequence. Such enhancers not only amplify but also can regulate expression of the rKGF<sub>des1-23</sub> polypeptide. Suitable

enhancer elements for use in mammalian expression systems are, for example, those derived from viruses that have a broad host range, such as the SV40 early gene enhancer, as described in Dijkema et al., *EMBO J.* 4:761 (1985), the enhancer/promoters derived from the LTR of the Rous Sarcoma Virus, as described in Gorman et al., *Proc. Natl. Acad. Sci.* 79:6777 (1982), and from human cytomegalovirus, as described in Boshart et al., *Cell* 41:521 (1985). Additionally, other suitable enhancers include those that can be incorporated into promoter sequences that will become active only in the presence of an inducer, such as a hormone, a metal ion, or an enzyme substrate, as described in Sassone-Corsi and Borelli, *Trends Genet.* 2:215 (1986); and Maniatis et al., *Science* 236:1237 (1987).

For expression in yeast cells, a yeast promoter is preferably used that contains an upstream activator sequence ("UAS") that permits regulated expression of the recombinant KGF<sub>des1-23</sub>. Regulated expression herein may be either positive or negative, thereby either enhancing or reducing transcription. In an alternative embodiment, a UAS can be absent, in which instance, constitutive expression of KGF<sub>des1-23</sub> would occur.

In another embodiment of the present invention, a transcription termination sequence is placed 3' to the translation stop codon of the KGF<sub>des1-23</sub> coding sequence. Thus, the terminator sequence, together with the promoter, flank the KGF<sub>des1-23</sub> coding sequence. Examples of transcription terminator sequences are the yeast-recognized sequences associated with the yeast glycolytic enzymes and those derived from SV40.

In one embodiment of the present invention, the recombinant KGF<sub>des1-23</sub> can be made to be secreted from the host cell into the growth media by creating chimeric DNA molecules that encode a fusion protein comprised of a leader sequence that contains a secretory signal sequence for secretion of KGF<sub>des1-23</sub>. For the purpose of the present invention, the signal sequences that are suitable for use herein are, for example, those derived from genes for secreted endogenous host cell proteins, such as for the yeast expression system, the yeast invertase gene, as described in European Patent No. 012 873 and Japanese Patent Application, Publication No. 62,096,086, the A-factor gene, as described in U.S. Pat. No. 4,588,684, the prepro  $\alpha$ -factor gene, as described in U.S. Pat. No. 4,870,008, and the interferon gene, as described in European Patent No. 060 057.

In a preferred embodiment of the present invention, a truncated yeast  $\alpha$ -factor leader sequence can be used. The truncated  $\alpha$ -factor leader sequence contains at least a portion of the signal "pre" sequence, and a portion of the "pro" sequence that contains a glycosylation site. Typically, the  $\alpha$ -factor leader sequences that can be employed herein include about 25 to about 50 amino acid residues of the N-terminal thereof, as described in U.S. Pat. Nos. 4,546,083 and 4,870,008, and in copending U.S. patent application Ser. No. 07/864,206 and European Patent Application, Publication No. 324 274. Also suitable for use herein are hybrid  $\alpha$ -factor leaders made with a "pre" sequence of a first yeast signal sequence, and a "pro" region from a second yeast  $\alpha$ -factor, as described in PCT Application, Publication No. WO 89/02463. In one embodiment of the present invention, the signal sequence contains a processing site 5' of the coding sequence of KGF<sub>des1-23</sub>, to allow cleavage thereof, either in vivo or in vitro. For mammalian expression, an example of a suitable leader sequence is the adenovirus tripartite leader that provides for secretion of an operably linked KGF<sub>des1-23</sub> protein in mammalian cells.

Prokaryotic leader sequences for directing the secretion of recombinant KGF<sub>der1-23</sub> that are suitable for use herein are those known in the art. For example, such leader sequences include those disclosed in U.S. Pat. No. 4,336,336, relating to "Fused Gene and Method of Making and Using the Same," to T. J. Silhavy et al., issued on Jun. 22, 1982, and U.S. Pat. No. 5,010,015, relating to "Recombinant DNA Molecules and Method for Protein Production," to Palva, I., issued on Apr. 23, 1991.

In another embodiment of the present invention, the expression vector can contain an origin of replication such that it can be maintained as a replicon, capable of autonomous replication and stable maintenance in a host. Such an origin of replication includes those that enable an expression vector to be reproduced at a high copy number in the presence of the appropriate proteins within the cell, for example, the 2 $\mu$  and autonomously replicating sequences that are effective in yeast, and the origin of replication of the SV40 vital T-antigen, that is effective in COS-7 cells.

Mammalian replication systems that are suitable for the present invention include those derived from animal viruses that require trans-acting factors to replicate. For example, the replication system of papovaviruses, such as SV40, as described in Gluzman, *Cell* 23:175 (1981), or polyomavirus that replicate to extremely high copy number in the presence of the appropriate vital T antigen. Additional examples include those derived from bovine papillomavirus and Epstein-Barr virus.

Additionally, the expression vector herein can have more than one replication system, thus, allowing it to be maintained, for example, in mammalian cells for expression and in a prokaryotic host for cloning and amplification. Examples of such mammalian-bacteria shuttle vectors include pMT2, as described in Kaufman et al., *Mol. Cell. Biol.* 9:946 (1989) and pHEBO, as described in Shimizu et al., *Mol. Cell. Biol.* 6:1074 (1986). Examples of yeast-bacteria shuttle vectors include YEp24, as described in Botstein et al., *Gene* 8:17-24 (1979); pCI/1, as described in Brake et al., *Proc. Natl. Acad. Sci. USA* 81:4642-4646 (1984); and YRp17, as described in Stinchcomb et al., *J. Mol. Biol.* 158:157 (1982).

The replicon herein may be either a high or low copy number plasmid. A high copy number plasmid will generally have a copy number ranging from about 5 to about 200, and typically about 10 to about 150. A host containing a high copy number plasmid will preferably have at least about 10, and more preferably at least about 20. Either a high or low copy number vector can be used herein, depending upon the effect of the vector and the recombinant KGF<sub>der1-23</sub> on the host. See e.g., Brake et al. (loc. cit.).

In another embodiment of the present invention, the expression vector can be made to integrate into the host cell genome as an integrating vector. The integrating vector herein contains at least one polynucleotide sequence that is homologous to the host cell genome that allows the vector to integrate. Preferably, the host cell contains two homologous sequences flanking the KGF<sub>der1-23</sub> coding sequence, as described in, for example, European Patent Application, Publication No. 127 328, relating to integrating vectors constructed with DNA from various *Bacillus* strains integrated into the *Bacillus* chromosome. Integrating vectors may also be comprised of bacteriophage or transposon sequences.

The homologous sequences, however, need not be linked to the expression vector. For example, an expression vector can be used that can integrate into the CHO genome via an

unattached dihydrofolate reductase gene. In a preferred embodiment, the homologous sequences flank the KGF<sub>der1-23</sub> coding sequence in the vector. Particularly useful homologous yeast genome sequences for the present invention are, for example, those disclosed in PCT Application, Publication No. WO 90/01800, and the *his4* gene sequences, as described in Genbank, accession no. J01331.

In another embodiment of the present invention, one or more selectable markers can be attached to other components in the expression vector to allow for the selection of the host cells that have been transformed. Selectable markers that can be expressed in a host cell include genes that can render the host cell resistant to drugs such as tunicamycin, G418, ampicillin, chloramphenicol, erythromycin, kanamycin (neomycin), and tetracycline, as described in Davies et al., *Ann. Rev. Microbiol.* 32:469 (1978). Selectable markers herein also include biosynthetic genes, such as those in the histidine, tryptophan, and leucine biosynthetic pathways, such as *ade2*, *his4*, *leu2*, *trp1*. Thus, for example, when a *leu*<sup>-</sup> host cell is used as recipient in transformation with an expression vector, and leucine is absent from the media, for example, only the cells that carry a plasmid with a *leu*<sup>+</sup> gene will survive.

In addition, selectable markers that provide the host cells with the ability to grow in the presence of toxic compounds, such as metal, is also suitable herein; for instance, the presence of *cup1* allows yeasts to grow in the presence of copper ions, as described in Butt et al., *Microbiol. Rev.* 51:351 (1987).

The method for construction of an expression vector for transformation of insect cells for expression of recombinant KGF<sub>der1-23</sub> herein is slightly different than that generally applicable to the construction of a bacterial expression vector, a yeast expression vector, or a mammalian expression vector. In an embodiment of the present invention, a baculovirus vector is constructed in accordance with techniques that are known in the art, for example, as described in Kitts et al., *BioTechniques* 14:810-817 (1993), Smith et al., *Mol. Cell. Biol.* 3:2156 (1983), and Luckow and Summer, *Virology* 17:31 (1989). In one embodiment of the present invention, a baculovirus expression vector is constructed substantially in accordance to Summers and Smith, Texas Agricultural Experiment Station Bulletin No. 1555 (1987). Moreover, materials and methods for baculovirus/insect cell expression systems are commercially available in kit form, for example, the MaxBac® kit from Invitrogen (San Diego, Calif.).

Briefly, a KGF expression cassette including the KGF<sub>der1-23</sub> coding sequence and optionally, a regulatory sequence, a selectable marker, etc., can be first constructed by insertion of the KGF<sub>163</sub> or KGF<sub>der1-23</sub> coding sequence into a baculovirus transfer vector that contains at least an essential polynucleotide sequence, as described in more detail below, and/or a polynucleotide sequence that is homologous to a portion of the baculovirus genome ("the baculovirus sequences") and is capable of homologous recombination therewith, for example, the polyhedron gene. Insertion therein can be engineered at a restriction enzyme site, as described in Miller et al., *Bioassays* 4:91 (1989). In a preferred embodiment, a KGF<sub>der1-23</sub> coding sequence is positioned downstream from the polyhedron promoter and flanked at both the 5' and the 3' end by polyhedron-specific sequences.

The KGF<sub>163</sub> coding sequence can be obtained by known recombinant techniques from cells that are known to express

KGF activity. The transfer vector containing the KGF<sub>des1-23</sub> coding sequence is co-transfected into host insect cells together with a mutant of wild-type baculovirus, so as to form a recombinant baculovirus. This mutant baculovirus lacks an essential polynucleotide sequence necessary for production of a functional recombinant virus. In this regard, a functional virus is one that is capable of independent replication in a host. A functional recombinant virus is obtained when the mutant baculovirus and the transfer vector carrying the KGF expression cassette both infect a host insect cell and recombine therein.

Recombinant baculovirus can be identified by known methods. For example, the wild-type viruses produce a polyhedron protein at very high levels in the nuclei of infected cells during a late stage of viral infection. Accumulated polyhedron protein forms occlusion bodies that also contain embedded viral particles. These occlusion bodies, up to 15 µm in size, are highly refractile, giving them a bright shiny appearance that is readily visualized under the light microscope. Cells infected with recombinant viruses lack occlusion bodies. Recombinant virus and wild-type virus can, therefore, be distinguished by plating the transfection supernatant or dilutions thereof onto a monolayer of insect cells by standard techniques. The plaques can then be screened under the light microscope for the presence, indicative of wild-type virus, or absence, indicative of recombinant virus, of occlusion bodies, as described in Vol. 2. of CURRENT PROTOCOLS IN MOLECULAR MICROBIOLOGY 16.8 F. M. Ausubel et al. eds, Supp. 10, Green Publisher Associates and Wiley Interscience (1990).

The functional recombinant baculovirus containing the KGF expression cassette obtained in this manner is suitable for transfection into new host insect cells for production of large quantities of the recombinant KGF<sub>des1-23</sub>. Recombinant KGF<sub>des1-23</sub> produced in this manner can be separated from KGF<sub>163</sub> based upon molecular weight differences, using known separatory techniques.

When making rather than purchasing a commercially available baculovirus/insect cell expression kit, the baculovirus transfer vector herein can be made by linking different components together, such as a promoter, a secretory leader sequence, and a transcription termination sequence. Alternatively, the transfer vector can be constructed such that a single copy of the KGF<sub>163</sub> coding sequence is operably linked to one or more regulatory elements, or multiple copies of the KGF<sub>des1-23</sub> coding sequence are each operably linked to its own set of regulatory elements. As a further alternative, multiple copies of the KGF<sub>des1-23</sub> coding sequence can be regulated by the same set of regulatory elements.

The transfer vector for the present invention may optionally contain an origin of replication so that it can be maintained as a replicon that is capable of stable maintenance in a host, such as a bacterium, for cloning and amplification. In a preferred embodiment of the present invention, the transfer vector for introducing KGF<sub>des1-23</sub> into AcNPV is pAc373. Other vectors, known to those of skill in the art, can also be used, including, for example, pVL985, which alters the polyhedrin start codon from ATG to ATT, and which introduces a BamHI cloning site 32 basepairs downstream from the ATT, as described in Luckow and Summers, *Virology* 17:31 (1989). After the expression vector is made, the vector is used to transform host cells for expression of recombinant KGF<sub>des1-23</sub>. The host cells that are suitable for use herein include prokaryotes and eukaryotes. The prokaryotes include Gram positive and Gram negative bacteria. The eukaryotes include fungi, yeast,

mammalian, and insect cells. The bacterial hosts include, for example, *Campylobacter*, *Bacillus*, *Escherichia*, *Lactobacillus*, *Pseudomonas*, *Staphylococcus*, and *Streptococcus*. Yeast hosts from the following genera may be utilized: *Candida*, *Hansenula*, *Kluyveromyces*, *Pichia*, *Saccharomyces*, *Schizosaccharomyces*, and *Yarrowia*. Mammalian cell lines suitable for use herein include, for example, many immortalized cell lines available from the American Type Culture Collection (ATCC), such as Chinese hamster ovary ("CHO") cells, HeLa cells, baby hamster kidney ("BHK") cells, monkey kidney cells ("COS"), and human hepatocellular carcinoma cells such as Hep G2. A number of insect cell hosts are also suitable for expression of the KGF fragment or analog, including, for example, *Aedes aegypti*, *Autographa californica*, *Bombyx mori*, *Drosophila melanogaster*, and *Spodoptera frugiperda*, as described in PCT Application, Publication No. WO 89/046699; Carbonell et al., *J. Virol.* 56:153 (1985); Wright *Nature* 321:718 (1986); Smith et al., *Mol. Cell. Biol.* 3:2156 (1983); and generally, Fraser, et al. in *vitro Cell. Dev. Biol.* 25:225 (1989).

Certain expression vectors, either extra-chromosomal replicons or integrating vectors, have been developed for transformation into certain hosts. A person of ordinary skill in the art would be able to adopt or adapt one or more of these developed vectors for use herein. For example, expression vectors have been developed for the following bacterial hosts that can be used herein: *Bacillus subtilis*, as described Palva et al., *Proc. Natl. Acad. Sci. USA* 79:5582 (1982); European Patent No. 036 259 U.S. Pat. No. 4,711,843 and European Patent Application, Publication No. 063 953; PCT Application, Publication No. WO 84/04541, U.S. Pat. No. 4,663,280; *Escherichia coli*, Shimatake et al., *Nature* 292:128 (1981); Amann et al., *Gene* 40:183 (1985); Studier et al., *J. Mol. Biol.* 189:113 (1986); European Patent No. 036 776 and European Patent Application, Publication Nos. 136 829 and 136 907; *Streptococcus cremoris*, Powell et al., *Appl. Environ. Microbiol.* 54:655 (1988); *Streptococcus lividans*, Powell et al., *Appl. Environ. Microbiol.* 54:655 (1988); *Streptomyces lividans*, U.S. Pat. No. 4,745,056.

Expression vectors that have been developed for yeasts that can be used herein include, for example, *Candida albicans* as described in Kurtz, et al. *Mol. Cell. Biol.* 6:142 (1986); *Candida maltosa* in Kunze, et al. *J. Basic Microbiol.* 25:141 (1985); *Hansenula polymorpha*, in Gleeson, et al. *J. Gen. Microbiol.* 132:3459 (1986); Roggenkamp et al. *Mol. Gen. Genet.* 202:302 (1986); *Kluyveromyces fragilis*, in Das, et al. *J. Bacteriol.* 158:1165 (1984); *Kluyveromyces lactis*, in De Louvencourt et al. *J. Bacteriol.* 154:737 (1983); Van den Berg et al. *Bio/Technology* 8:135 (1990); *Pichia guilliermondii*, in Kunze et al. *J. Basic Microbiol.* 25:141 (1985); *Pichia pastoris*, in Cregg et al. *Mol. Cell. Biol.* 5:3376 (1985); U.S. Pat. No. 4,837,148 and U.S. Pat. No. 4,929,555; *Saccharomyces cerevisiae*, in Hinnen et al. *Proc. Natl. Acad. Sci. USA* 75:1929 (1978); Ito et al. *J. Bacteriol.* 153:163 (1983); *Schizosaccharomyces pombe*, in Beach and Nurse, *Nature* 300:706 (1981); and *Yarrowia lipolytica* in Davidow, et al., *Curr. Genet.* 10:380-471 (1985); Gaillardin et al., *Curr. Genet.* 10:49 (1985).

The transformation procedures suitable for use herein are those known in the art and include, for example, first treating the bacterial cells with CaCl<sub>2</sub> or other agents, such as divalent cations and DMSO, and incubating the treated cells with the KGF<sub>des1-23</sub> coding sequence. DNA can also be introduced into bacterial cells by electroporation. The exact transformation procedure varies with the bacterial species to be transformed as described in, for example, Masson et al.,



*FEMS Microbiol. Lett.* 60:273 (1989); Palva et al., *Proc. Natl. Acad. Sci. USA* 79:5582 (1982); European Patent Application, Pub. Nos. 036 259 and 063 953; PCT Application, Publication No. WO84/04541, for Bacillus; Miller et al., *Proc. Natl. Acad. Sci.* 85:856 (1988); Wang et al., *J. Bacteriol.* 172:949 (1990), for Campylobacter; Cohen et al., *Proc. Natl. Acad. Sci.* 69:2110 (1973); Dower et al., *Nucleic Acids Res.* 16:6127 (1988); Kushner "An improved method for transformation of *Escherichia coli* with ColEI-derived plasmids", in *GENETIC ENGINEERING: PROCEEDINGS OF THE INTERNATIONAL SYMPOSIUM ON GENETIC ENGINEERING*, H. W. Boyer and S. Nicosia, eds. Amsterdam and New York: Elsevier and North Holland Biomedical Press (1978); Mandel et al., *J. Mol. Biol.* 53:159 (1970); Taketo, *Biochim. Biophys. Acta* 949:318 (1988), for *Escherichia*; Chassy et al., *FEMS Microbiol. Lett.* 44:173 (1987), for *Lactobacillus*; Fiedler et al., *Anal. Biochem.* 170:38 (1988), for *Pseudomonas*; Augustin et al., *FEMS Microbiol. Lett.* 66:203 (1990), for *Staphylococcus*; Barany et al., *J. Bacteriol.* 144:698 (1980); Harlander "Transformation of *Streptococcus lactis* by Electroporation", in *STREPTOCOCCAL GENETICS*, J. Ferretti and R. Curtiss III, eds (1987); Perry et al., *Infect. Immun.* 52:1295 (1981); Powell et al., *Appl. Environ. Microbiol.* 54:655 (1988); Somkuti et al., *Proc. 4th Eur. Cong. Biotechnology* 1:412 (1987), for *Streptococcus*.

For yeast, the transformation procedures that can be used herein include electroporation, as described in "Guide to Yeast Genetics and Molecular Biology," Vol 194 METHODS IN ENZYMOLOGY, C. Guthrie and G. R. Pink. (Academic Press 1991). Other procedures include the transformation of spheroplasts or the transformation of alkali cation-treated intact cells. Such procedures are described in, for example, Kurtz et al., *Mol. Cell. Biol.* 6:142 (1986); Kunze et al., *J. Basic Microbiol.* 25:141 (1985), for *Candida*; Gleeson et al., *J. Gen. Microbiol.* 132:3459 (1986); Roggenkamp et al., *Mol. Gen. Genet.* 202:302, for *Hansenula* (1986); Das et al., *J. Bacteriol.* 158:1165 (1984); De Louvencourt et al., *J. Bacteriol.* 154:1165 (1983); Van den Berg et al., *Bio/Technology* 8:135 (1990) for *Kluyveromyces*; Cregg et al., *Mol. Cell. Biol.* 5:3376 (1985); Kunze et al., *J. Basic Microbiol.* 25:141 (1985); U.S. Pat. No. 4,837,148 and U.S. Pat. No. 4,929,555, for *Pichia*; Hinnen et al., *Proc. Natl. Acad. Sci. USA* 75:1929 (1978); Ito et al., *J. Bacteriol.* 153:163 (1983), for *Saccharomyces*; Beach and Nurse *Nature* 300:706 (1981), for *Schizosaccharomyces*; Davidow et al., *Curr. Genet.* 10:39 (1985); Gaillardin et al., *Curr. Genet.* 10:49 (1985), for *Yarrowia*.

For example, for mammalian cell systems, such methods include dextran-mediated transfection, calcium phosphate precipitation, polybrene-mediated transfection, protoplast fusion, electroporation, encapsulation of the KGF<sub>des1-23</sub> polynucleotide in liposomes, and direct microinjection of the DNA into nuclei.

Immunoassays and activity assays that are known in the art can be utilized herein to determine if the transformed host cells are expressing the desired KGF fragment. For example, for detection of intracellular production of KGF<sub>des1-23</sub> by transformed host cells, an immunofluorescence assay can be performed on the transformed host cells without separating the KGF fragments from the cell membrane. In this assay, the host cells are first fixed onto a solid support, such as a microscope slide or microtiter well. Next, the fixed host cells are exposed to an anti-KGF antibody. Preferably, to increase the sensitivity of the assay, the fixed cells are exposed to a second antibody, that is labelled and binds to the anti-KGF antibody. For instance, the secondary antibody may be

labelled with an fluorescent marker. The host cells which express the KGF fragments will be fluorescently labelled and can be visualized under the microscope.

In another embodiment of the present invention, the recombinant KGF<sub>des1-23</sub> polypeptide can be expressed as a fusion protein in any of the above expression systems. For example, for yeast expression, in the construction of an expression vector, a DNA sequence encoding the N-terminal portion of an endogenous yeast protein, or other stable protein, can be fused to the 5' end of the coding sequence of KGF<sub>des1-23</sub>. The DNA sequence at the junction of the two amino acid sequences optionally encodes a cleavable site, as discussed in European Patent Application, Publication No. 196 056.

An example of a DNA sequence that encodes the N-terminal portion of the fusion protein is the sequence encoding at least a portion of yeast or human superoxide dismutase ("SOD") for expression in yeast. Another example is the sequence encoding at least a portion of the ubiquitin protein, preferably, containing the sequence that encodes the cleavage site or its processing enzyme, the ubiquitin-specific processing protease. See, e.g., PCT Application Publ. No. WO 88/024066.

KGF toxin conjugates suitable for use herein can be produced by methods known in the art, for example, U.S. Pat. Nos. 4,771,128, 4,808,705, and 4,894,443 and PCT Application, Publ. No. WO 92/04918.

The present invention includes variants and modifications of the above that do not substantially alter the nature and activity of the fragment, conjugate, therapeutic composition, vector, host, and methods of use and that are apparent to a person of ordinary skill in the art.

## EXAMPLES

The examples presented below are provided to demonstrate the present invention, and are not to be construed as limiting the invention in any way.

### Example 1

#### The Production of a KGF Coding Sequence Linked to a Signal Peptide

PCR techniques were applied to the construction of the KGF<sub>163</sub> coding sequence linked to a signal peptide, SEQ ID NO: 1. The signal peptide was one that was associated with the native KGF<sub>163</sub> coding sequence, as described in Finch et al., *Science* 245:752-755 (1989).

The following primers were used to clone the KGF<sub>163</sub> coding sequence and its signal peptide from a human kidney cDNA library:

SEQ ID NO: 4 (sense primer)

PstI Met His Lys Trp Ile Leu\*  
5' AGATCTCTGCAGCTATA ATG CAC AAA TGG ATA CTG 3'

SEQ ID NO: 6 (antisense primer)

NotI Stop Thr Ile Ala Met Pro Leu Phe\*\*  
5' AGATCTGCGGCCGC TTA AGT TAT TGC CAT ACG AAG AAA 3'

\*The peptide sequence is listed in the Sequence Listing as SEQ ID NO: 3.

\*\*As encoded by the reverse complement of the primer, and the peptide sequence is listed as SEQ ID NO: 5.

As shown in the sequences above, in addition to the KGF<sub>163</sub> coding sequence and the signal peptide, additional nucleotides were incorporated at the 5' and the 3' ends, respectively. The additional nucleotides include a PstI site at the 5' end, and a NotI site at the 3' end of the respective

primers, to facilitate cloning of the KGF<sub>163</sub> a coding sequence and its signal peptide into the desired insect cell transfer vector. (See Example 2 below.)

To generate the desired KGF<sub>163</sub> DNA molecule, 1 µl of each primer, at a final concentration of 1 µM, was added to 2.5 µl or approximately 10 ng. of human kidney cDNA library, obtained from Clontech, Palo Alto, Calif., U.S.A. The following reagents from the Perkin-Elmer PCR kit (Norwalk, Conn., U.S.A.) were also added to the primer/cDNA library: 16 µl of 1.25 mM dNTP, containing equimolar amounts of each of dATP, dTTP, dCTP and dGTP, 10 µl of 10x buffer, 0.5 µl of Taq polymerase, at 5 units/ml; and 69 µl of water. PCR was performed on a DNA Thermal Cycler, from Perkin Elmer, Norwalk, Conn., U.S.A., as follows: 94° C. for 1 minute, 55° C. for 2 minutes, and 72° C. for 3 minutes. This temperature cycle was repeated 30 times. The resulting PCR product was treated with DNA PolI (Klenow) and then phenol/chloroform extracted, chloroform extracted, and ethanol precipitated, respectively.

The PCR product was digested with PstI and NotI and gel purified before ligation.

#### Example 2

##### The Production of a KGF Insect Cell Expression Vector

The purified PCR product, comprising the KGF<sub>163</sub> coding sequence and its signal peptide, was inserted into a baculovirus transfer vector. The specific baculovirus used is *Autographa californica* nuclearpolyhedrosis virus (AcNPV).

The purified PCR product from Example 1, digested with restriction enzymes PstI and NotI, was ligated to pAcC13, which was also digested with PstI and NotI. pAcC13 is a baculovirus transfer vector and is depicted in FIG. 2. This plasmid was derived from pAcC12, as described in Munemitsu et al., *Mol. Cell. Biol.* 10:5977-5982 (1990) and a derivative of the pVL941 transfer vector, the construction of which is described in Quilliam et al., *Mol. Cell. Biol.* 10:2901-2908 (1990); Luckow et al., *Virol.* 170:31-39 (1989); and Smith et al., *Mol. Cell. Bio.* 3(12): 2156-2165 (1983).

The transfer vector further contains a polylinker conveniently placed between the baculovirus polyhedrin promoter and terminator. The DNA sequence of the polylinker comprises unique restriction sites. Thus, by digesting the transfer vector with the PstI and NotI sites within the polylinker, the KGF<sub>163</sub> coding sequence and the signal peptide can be inserted between the polyhedrin promoter and terminator. In addition, the transfer vector contains sequences from the essential gene of AcNPV baculovirus. The transfer vector was named pAc/KGF.

#### Example 3

##### The Production of a Recombinant Baculovirus Capable of Expressing the KGF Coding Sequence

The transfer vector with the KGF<sub>163</sub> coding sequence and its signal peptide insert was transfected together with a mutant baculovirus into *Spodoptera frugiperda*, SF9 cells. The mutant baculovirus is a derivative of AcNPV that lacks a functional essential gene. This mutant baculovirus must recombine with the transfer vector to produce a viable baculovirus. To increase the number of recombinants, the mutant baculovirus was linearized with BsuI. In this regard, several BsuI sites were incorporated into the mutant baculovirus for this purpose. This mutant baculovirus is similar to the baculovirus described in Kitts et al., *Biotechniques* 14(5):810-817 (1993).

##### A. Preparation of Recombinant Baculovirus

First, 1×10<sup>6</sup> SF9 cells were seeded per well in a 6-well plate containing 2 ml of a complete TNMFH medium. The cells were incubated for at least 30 minutes at room temperature to allow the cells to attach to the plate. The complete TNMFH medium contained GRACE'S medium obtained from JRH Biosciences, Lenexa, Kans., U.S.A. and supplemented with 10% (v/v) fetal bovine serum (56° C. heat inactivated for 30 minutes), 3% (w/v) Yeastolate (Difco, Detroit, Mich., U.S.A.), and 1% (v/v) Fungi-BACT (Irvine Scientific, Santa Ana, Calif., U.S.A.).

For addition to each of the above wells, the following transfection mixture was initially separately prepared by first, adding 0.5 ml of GRACE'S medium containing no supplement into a sterile 1.5 ml eppendorf tube; next, adding 0.5 µg of linearized mutant baculovirus DNA and, approximately, 2-3 µg of the transfer vector containing the KGF<sub>163</sub> coding sequence and the signal peptide insert. This is approximately a 4:1 ratio of transfer vector to mutant baculovirus. Finally, the cationic liposome solution, BRL catalog #8282SA (from BRL, Gaithersburg, Md., U.S.A.) was mixed thoroughly, and 10 µl of this liposome solution were added to the baculovirus mixture. This transfection mixture was incubated at room temperature for 15 minutes.

Before the transfection mixture was added to the cells in the wells, the TNMFH medium was removed therefrom, and the cells were washed with 1-2 ml of GRACE'S medium without supplements. When the transfection mixture was finally prepared, as described above, all the media were removed from the SF9 cells, and the transfection mixture was added dropwise to the cells. The 6-well plate was then covered with parafilm to reduce evaporation of the transfection mixture. The plate was rocked slowly at room temperature for approximately four hours on a Bellco® catalog no. #774020020, Vineland, N.J., U.S.A., side/side rocking platform at setting 2.5.

After the incubation of the cells with the transfection mixture, 0.5 ml of complete TNMFH medium was added to the cells and the mixture was incubated at 27° C. in a humidified chamber (92%) for 48 hours. Thereafter, the medium containing recombinant baculovirus was removed from the cells and stored at 4° C. until the plaque assay for recombinant virus isolation was performed. This medium constituted the primary source of the recombinant virus.

After the medium was removed from the cells, 2 ml of complete TNMFH were added to the cells, and the cells were further incubated in a humidified chamber (92%) at 27° C. for another 48-72 hours. This final step was performed to provide a back-up source of recombinant virus and to provide a visual record of the viral infection.

##### B. Plaque Purification of the Recombinant Baculovirus

The recombinant KGF baculovirus prepared as above were plaque-purified according to the following steps:

First, 4 ml of SF9 cells at 5×10<sup>5</sup> cells/ml in GRACE'S medium were plated on 60 mm LUX dishes, catalog #5220. The cells were incubated at room temperature for 20-30 minutes to allow the cells to adhere to the plate. In the meantime, the medium containing the primary source of recombinant virus was diluted into 2 ml of TNMFH medium at 1:10, 1:50, 1:100, and 1:200 dilutions. After the cells were allowed to adhere, the medium was aspirated from the adherent cells, and the various dilutions of the recombinant virus were added quickly so as not to allow the cells to dry. The cells were then incubated for 1 hour at 27° C. in a humidified chamber (92%).

Ten minutes before the incubation was complete, an agarose solution was prepared. A 2x concentration of



GRACE'S medium supplemented as before was heated to 37° C. Only the amount used for the assay was heated; otherwise, proteins may precipitate upon repeated heating. When the 2x medium was warm, a 3% (w/v) Sea Plaque agarose mixture in water was melted in a microwave and immediately mixed 1:1 with the 2x medium. The agarose solution was then allowed to cool at room temperature for several minutes.

The viral medium was aspirated from the cells in the dishes by tilting the dishes slightly on the hood rim. The dishes were then drained for a few seconds and aspirated again to remove as much liquid as possible. This second aspiration step was included to reduce the likelihood of virus spreading and causing indistinct plaques. Six dishes or fewer were handled at a time to avoid drying the cells. The aspirated dishes were never left exposed with the lids off for more than a few seconds.

Next, 4 ml of the agarose solution were added to each dish and left undisturbed for 15 minutes. The agarose overlay was dried by lifting the lids to the side of the plate to permit the agarose to dry for approximately 25 minutes. Then, the dishes were covered with the lids, and the cells were incubated in a humidified chamber (92%) for 4 days at 27° C.

To facilitate visualization of the plaques, the dishes were stained with 2 ml per dish of 25% (w/v) Sea Plaque agarose in complete TNMFH medium with 0.01% (v/v) neutral red, from Sigma, St. Louis, Mo., U.S.A. The agarose overlay was dried at room temperature for about one hour with the lids on, before the dishes were returned to a humidified chamber (92%) at 27° C. for 3-4 hours. The neutral red dye was incorporated by the viable but not the dead cells.

When the plaques were well-resolved, 7 individual plugs were picked with a sterile Pasteur pipet and each was transferred to 1 ml of complete TNMFH. The plugs were incubated for 2 days at room temperature. The plugs were then vortexed, and the plaque assay was repeated with 50 µl of this solution.

#### C. Expansion of the Plaque Purified Baculovirus

To expand the viral titers, 3-4 plugs of each baculovirus clone from the second round of plaque purification were placed directly onto cultures of SF9 cells. The cells were plated at  $2.5 \times 10^5$ /well in 6-well plates with 2.5 ml of complete TNMFH medium. The cells and virus were then incubated for approximately 4 hours at 27° C. in a humidified chamber (92%).

For the second round of expansion, all the virus from the 6-well plates were transferred to 10 cm dishes. The 10 cm dishes were plated with  $7.5 \times 10^6$  cells in 7.5 ml of complete TNMFH media. The cells and virus were incubated for 48-72 hours at 27° C. in a humidified chamber (92%).

After this infection, the cells were thoroughly screened for any wild type virus contamination that appeared as infected cells containing occlusion bodies. No occlusion bodies were observed. The resulting recombinant baculovirus was named KGF-5 and was deposited with the American Type Culture Collection ("ATCC") at 12301 Parklawn Drive, Rockville, Md., U.S.A. 20852-1776 on 17 Jun. 1993, Accession No. VR2411.

Supernatant from the second baculovirus expansion round was tested for KGF bioactivity. Also, the crude supernatant was run on a sodium dodecyl sulfate polyacrylamide gel electrophoresis ("SDS-PAGE") gel, and stained with Coomassie blue to confirm the expression of KGF.

#### Example 4

##### Baculovirus Expression of a KGF Coding Sequence

SF9 insect cells were infected with the recombinant baculovirus, as described in Example 3, to provide a large amount of recombinant KGF fragments for purification and analysis.

More specifically, SF9 cells were diluted to a cell density of  $1 \times 10^6$  cells/ml in Excell-400 (JRH Biosciences, Lenexa, Kans. U.S.A.). The cells were seeded into either a 1 L or 2 L shake flask with 300 ml or 500 ml of medium, respectively. Virus from the second expansion (described in Example 3, section C) was used to inoculate the cells at 10% (v/v) dilution. The cells and virus were shaken at approximately 131 rpm with the caps of the shake flasks loosened to provide an adequate air supply. The cells were incubated at 27° C. for 48 hrs.

Conditioned medium from the incubated cell culture above was collected by centrifuging the cell culture at  $15,000 \times g$  for 10 minutes at 4° C. to remove the cells. Next, the conditioned medium was filtered with an 0.8 µm cellulose nitrate filter (Millipore). Approximately 5 liters of this conditioned medium was concentrated to 200 ml using a filtron cassette system (Omega membrane), having a 3 kD molecular weight cut-off. The pH of the retentate was adjusted to 7.2 with 1N NaOH and the small amount of precipitate was removed by centrifugation at  $10,000 \times g$  for 20 minutes at 4° C.). The concentrated conditioned medium was then immediately applied to a Heparin Sepharose® ("HS") resin column.

#### Example 5

##### Purification of a KGF<sub>der1-23</sub> Fragment

Recombinant KGF<sub>der1-23</sub> from the concentrated conditioned medium described in Example 4 was purified by HS affinity chromatography, followed by Mono S cation exchange chromatography. The recombinant KGF<sub>der1-23</sub> bound to the HS was eluted using step-wise salt gradient. Next, the recombinant KGF<sub>der1-23</sub> eluted from the HS column was bound to the Mono S cation column and was eluted using a linear salt gradient.

Further details of the procedure utilized are described below.

##### A. Heparin Sepharose® Affinity Chromatography

First, the concentrated conditioned medium from Example 4 was allowed to run for approximately 2 hours at 4° C. through a 30 ml bed of HS resin. The column was equilibrated in a buffer containing 150 mM NaCl, and 10 mM Tris-HCl at pH 7.3. Once the concentrated conditioned medium was loaded, the column was washed extensively with the equilibration buffer until the absorbance at 280 nm returned to baseline. Then, protein was eluted from the HS column with an increasing step-wise NaCl gradient. The NaCl concentrations were 0.45M, 1M, and 2M NaCl, in 10 mM Tris-HCl at pH 7.3. The flow rate of the column during elution was approximately 90 ml/hr and 3-ml size fractions were collected.

The fractions were tested for KGF bioactivity utilizing Balb/Mk cells. The assay is described in section C below. The fractions with the highest bioactivity were eluted with 1M NaCl and were pooled. Before the pooled fractions were loaded onto the next column, the fractions were diluted five-fold with 10 mM Tris-HCl at pH 7.2 to a final salt concentration of 0.2M NaCl.

##### B. Mono S Cation Exchange Chromatography

The pooled fractions eluted from the HS column were loaded with a Super loop onto a Mono S column linked to

an FPLC system (Pharmacia, Piscataway, N.J.). The Mono S cation exchange column was equilibrated with 10 mM Tris-HCl at pH 7.2. When the pooled fractions were loaded, the column was washed extensively at a flow rate of 1 ml/min. with the equilibration buffer until the absorbance returned to baseline. Then, protein was eluted from the column with a linear NaCl gradient, 0.2M to 1M NaCl in 10 mM Tris-HCl at pH 7.3 at a flow rate of 1 ml/min., and 1 ml fractions were collected.

Two major protein peaks of activity were found that eluted at about 0.55M NaCl and 0.60M NaCl. Fractions across the protein peaks were assayed for bioactivity and subjected to SDS-PAGE analysis. The results of the gel analysis (not illustrated) showed that protein from 0.55M and 0.60M NaCl fractions exhibited an apparent molecular weight of 27 kD and 18 kD, respectively.

When the bioactivity of the fractions was determined, the 18 kD protein was found to exhibit at least a 2-, but more particularly a 7-10 fold more activity than the 27 kD protein, or than the positive control, the acidic fibroblast growth factor ("aFGF"), as shown in FIG. 5. The bioactivity of the fractions were tested using Balb/Mk cells. (See section C, below.)

To determine the amino acid sequence of the eluted protein, two 100 pmole samples of the 27 kD and 18 kD proteins were subjected to Edman degradation after centrifugal adsorption to polyvinylidene difluoride (PVDF, Applied Biosystems Prospin). The samples were loaded onto an Applied Biosystems 470A or 473A protein sequencer. Twenty rounds of Edman degradation were carried out using standard software and chemicals supplied by Applied Biosystems, and identifications of PTH-amino acids were made with an automated on-line HPLC system (FFH analyzer 120A, Applied Biosystems).

Using standard methodology well known in the art, an unambiguous amino acid sequence was established for positions 1 to 20 of the N-terminal of the 18 kD protein, which is KGF<sub>des1-23</sub>. The sequence was as follows:

S Y D Y M E G G D I R V R R L F X R T Q (SEQ ID NO: 7). The N-terminal sequence of the 27 kD protein is identical to the N-terminal sequence of KGF<sub>163</sub>.

#### C. KGF Bioactivity Assay Utilizing Balb/Mk Cells

KGF bioactivity was assessed by the ability of the fractions to promote growth of BALB/C-Mk cells.

Stock cultures of Balb/Mk cells were grown and maintained in low calcium Dulbecco's modified Eagle medium supplemented with 10% fetal bovine serum, 0.25 µg/ml fungizone, and 10 ng/ml aFGF. The cells were incubated at 37° C. in a 10% CO<sub>2</sub> atmosphere with 99% humidity. For the bioactivity assay, the cells were seeded in 12-well plates at a density of 5×10<sup>3</sup> cells per well in 1 ml of medium as described above for the stock cultures, and as described in Gospodarowicz et al., *J. Cell. Physiol.* 142: 325-333 (1990).

Ten microliter aliquots of the desired column fractions were diluted into 1 ml of 0.2% (w/v) gelatin in phosphate buffered saline ("PBS"). Ten microliters of this dilution were added to Balb/Mk cells seeded in 12-well cluster plates containing 22 mm webs, at 5×10<sup>3</sup> cells per well, and a 10 µl aliquot of either the diluted column fractions or medium containing 10 ng aFGF were added to the cells every other day.

After five days in culture, the cells were trypsinized and the final cell density was determined using a Coulter® counter. The cells were released from the plates by replacing the culture medium with a solution containing 0.9% NaCl,

0.01M sodium phosphate (pH 7.4), 0.05% trypsin, and 0.02% EDTA (STV). The cells were incubated in this solution for 5-10 minutes at 37° C., and then the stock culture medium was added to the cells. The cells were then counted using a Coulter® counter (Coulter Electronics, Hialeah, Fla., U.S.A.). Results shown in FIG. 4 demonstrates the effect of different dilutions of rKGF<sub>des1-23</sub> and rKGF<sub>163</sub> on Balb/Mk cells as compared to aFGF. The figure legend for FIG. 4 is as follows: ○=KGF<sub>des1-23</sub>, ●=KGF<sub>163</sub>, and □=aFGF.

The final cell density was graphed as a function of protein concentration. The protein concentration is graphed on a log scale. The protein concentration was determined by Bradford assay according to instructions accompanying the protein assay kit from BIORAD (Richmond, Calif., U.S.A.).

The ED<sub>50</sub> was calculated by (a), dividing in half the difference between the lowest and highest cell density value of the curve; and (b), determining from the graph what protein concentration corresponds to that cell density number obtained in (a). According to FIG. 4, the ED<sub>50</sub> for aFGF was found to be approximately 500 pg/ml, that for rKGF<sub>163</sub> was about 250 pg/ml, and that for KGF<sub>des1-23</sub> was about 24 pg/ml. The results of this assay demonstrate that rKGF<sub>des1-23</sub> was about 10-fold more active than rKGF<sub>163</sub>.

The exact ED<sub>50</sub> for the proteins may vary from assay to assay, but the ratio of the activity between the long and short form of KGF is at least 2 fold, preferably, at least a 7-fold, more preferably, about 7-10 fold, even more preferably, at least 10 fold. Even a 50-fold difference in activity had been observed.

For example, the bioactivity assay was repeated with different or with the same preparations of rKGF<sub>163</sub> and rKGF<sub>des1-23</sub>. The results of one of the assays showed that the ED<sub>50</sub> of rKGF<sub>163</sub> and rKGF<sub>des1-23</sub> were 600 pg/ml, and 80 pg/ml, respectively, about a 7-fold increase in activity. Another assay demonstrated a 50-fold difference between the long and short form of KGF. In this later assay, KGF<sub>des1-23</sub> exhibited an ED<sub>50</sub> of 30 pg/ml, and KGF<sub>163</sub> exhibited an ED<sub>50</sub> of 1500 pg/ml. In view of the fact that the last bioassay was done two (2) months after KGF isolation, the data suggest that upon storage, KGF<sub>163</sub> lost its bioactivity faster than KGF<sub>des1-23</sub>.

#### D. Absence of KGF Bioactivity on ABAE or ACE Cells

KGF can be characterized by its lack of activity on vascular endothelial cells derived from large vessels ("ABAE") or capillary cells ("ACE") as compared with basic FGF ("bFGF"). Stock cultures of ABAE and ACE cells were grown and maintained in low calcium Dulbecco's modified Eagle medium supplemented with 10% bovine serum, 0.25 µg/ml fungizone, and 2 ng/ml bFGF. The cells were incubated at 37° C. with a 10% CO<sub>2</sub> concentration and 99% humidity.

In the mitogenic assay, either 10<sup>4</sup> ABAE or 5×10<sup>3</sup> ACE cells were plated per well in 12-well plates in stock culture medium, as described in Gospodarowicz et al., *Proc. Natl. Acad. USA* 73:4120-4124 (1976); Gospodarowicz et al., *J. Cell. Physiol.* 127:121-136 (1976); and Gospodarowicz et al., *Proc. Natl. Acad. USA* 86:7311-7315 (1989). Ten microliter aliquots of the fractions to be tested were diluted into 1 ml of 0.2% (w/v) gelatin in phosphate buffered saline ("PBS"). Ten microliters of this dilution was added to cells seeded in 12-well cluster plates. A 10 µl aliquot of either the diluted purified rKGF medium containing fractions or 2 ng bFGF was added every other day.

After five days in culture, the cells were trypsinized and the final cell density was determined using a Coulter®

counter. The cells were released from the plates by replacing the medium with a solution containing 0.9% NaCl, 0.01M sodium phosphate (pH 7.4), 0.05% trypsin, and 0.02% EDTA (STV). The cells were incubated in this solution for 5–10 minutes at 37° C., and then the stock culture medium was added to the cells. The cells then were counted using a Coulter® counter (Coulter Electronics, Hialeah, Fla., U.S.A.).

Results shown in FIG. 5 demonstrates the lack of activity of both rKGF<sub>163</sub> and rKGF<sub>des1-23</sub> on ABAE cells and ACE cells, in contrast to bFGF. Similar observations were made using vascular smooth muscle cells, corneal endothelial cells, ovarian granulosa cells, and BkH-21 fibroblasts. Although bFGF did stimulate the proliferation of these various cell types, neither rKGF<sub>163</sub> or rKGF<sub>des1-23</sub> had any bioactivity.

#### Example 6

##### Construction of a KGF<sub>des1-23</sub> Yeast Vector for Secretion by Yeast Cells

This example describes the construction of a yeast expression vector for secretion of KGF<sub>des1-23</sub> by transformed yeast cells in accordance with the present invention.

Specifically, the components of the expression vector construct: KGF<sub>des1-23</sub> coding sequence operably linked, at its 5' end, to a glyceraldehyde-3-phosphate dehydrogenase ("GAPDH") promoter and the coding sequence of the first 35 amino acid residues of the *Saccharomyces cerevisiae*

SEQ ID NO: 12 (sense)

Pro Ala Lys Arg Ser Tyr Asp Tyr Met Glu Gly Gly\*  
5' CCG CCG GCT AAG CGA AGT TAT GAT TAC ATG GAA GGA GGG  
Bpu1102I

SEQ ID NO: 14 (antisense)

Stop Thr Ile Ala Met Pro Leu Phe His\*\*  
5' GGT GGT GTC GAC TTA AGT TAT TGC CAT AGG AAG AAA GTG 3'  
SalI

\*The peptide sequence is listed as SEQ ID NO: 11.

\*\*As encoded by the reverse complement of the primer, and the peptide is listed as SEQ ID NO: 13.

prepro  $\alpha$ -factor leader sequence and, at its 3' end, to the *Saccharomyces cerevisiae*  $\alpha$ -factor terminator.

The fragments for this construct:

- (1) a BamHI/Bpu1102I fragment containing the GAPDH promoter and the truncated prepro  $\alpha$ -factor leader containing a functional signal peptide;
- (2) a Bpu1102I/SalI fragment encoding KGF<sub>des1-23</sub>; and
- (3) a BamHI/SalI vector fragment of the yeast expression vector, pBS24.1, containing the  $\alpha$ -factor terminator, leucine, and uracil yeast selectable markers, and 2 $\mu$  sequences as an origin of replication.

##### A. Construction of the BamHI/Bpu1102I Promoter/Leader Fragment

The source of the BamHI/Bpu1102I fragment: by PCR using pGAI7 as a template. The plasmid pGAI7 is described in European Patent Application, Publication No. 324 274. This plasmid contains the same BamHI expression cassette as pYGAI7, which is available at the ATCC as Accession No. 67597. Plasmid pGAI7 contains approximately 400 bp GAPDH promoter fragment, as described in Travis et al., *J. Biol. Chem.* 4384–4389 (1985), and the prepro  $\alpha$ -factor leader sequence that encodes the N-terminal amino acid residues 1–35.

The primer for the PCR of BamHI/Bpu1102I promoter/leader fragment:

SEQ ID NO: 8 (sense)

BamHI  
5' GGTGGTGGATCCCGAGCTTAGTTCATAGGTCC 3'

SEQ ID NO: 10 (antisense)

His Gln Asn Val Phe Arg Lys Ala Pro Ile Gln Ala\*  
5' GTG TTG GTT AAC GAA TCG CTT AGC CGG AAT TTG TGC 3'  
Bpu1102I

\*As encoded by the reverse complement of the primer, the peptide sequence is listed in the Sequence Listing as SEQ ID NO: 9.

Reagents for generating the BamHI/Bpu1102I fragments by PCR: 1  $\mu$ l of each primer (100 pmol/ $\mu$ l); 1  $\mu$ l of the template, pGAI7 at ~500  $\mu$ g/ml, and the following reagents from Perkin Elmer PCR kit (Norwalk, Conn.): 8  $\mu$ l of dNTP (1.25 mM each of dATP, dTTP, dCTP, and dGTP), 10  $\mu$ l of 10x buffer, 0.5  $\mu$ l of Taq polymerase, and 63.5  $\mu$ l of water. The temperature cycle for PCR: 94° C. for 1 minute, 37° C. for 2 minutes, and 72° C. for 3 minutes. Number of repetitions of this temperature cycle: 30. Purification of the PCR product of ~414 bp by gel purification, and by the use of a GeneClean kit by bio101, La Jolla, Calif., U.S.A.

##### B. Construction of the Bpu1102I/SalI KGF<sub>des1-23</sub> Coding Sequence Fragment

The origin of the Bpu1102I/SalI KGF<sub>des1-23</sub> coding sequence fragment: by PCR using the pAcc/KGF baculovirus transfer vector described in Example 1 and 2.

The primers for PCR of the Bpu1102I/SalI KGF<sub>des1-23</sub> coding sequence:

Reagents for generating the Bpu1102I/SalI KGF<sub>des1-23</sub> coding sequence by PCR: 1  $\mu$ l of each primer (100 pmol/ $\mu$ l), 1  $\mu$ l of the template, pAcc/KGF, at 500  $\mu$ g/ml, and the following reagents from Perkin Elmer PCR kit (Norwalk, Conn., U.S.A.): 8  $\mu$ l of dNTP (1.25 mM each of dATP, dTTP, dCTP, dGTP), 10  $\mu$ l of 10x buffer, 0.5  $\mu$ l of Taq polymerase, and 63.5  $\mu$ l of water. The temperature cycle for PCR: 94° C. for 1 minute, 37° C. for 2 minutes, and 72° C. for 3 minutes. Number of repetitions of this temperature cycle: 30. Purification of the PCR product of ~414 bp by gel purification, and by use of a GeneClean kit by bio101, La Jolla, Calif., U.S.A.

##### C. Construction of the BamHI/SalI Vector Fragment

The source of the BamHI/SalI vector fragment: by digesting the yeast expression vector pBS24.1 with BamHI and SalI.

pBS24.1 is a yeast expression vector that is derived from pAB24. pBS24.1 contains the  $\alpha$ -factor terminator; the 2 $\mu$  sequences, as a yeast origin of replication; selectable markers for uracil and leucine in yeast; and an ampicillin resistance gene, effective in *E. coli*. This yeast expression vector also contains a human FGF coding sequence which can be excised by digestion with BamHI and SalI enzymes and isolating the larger vector fragment.

Plasmid pAB24 is a yeast shuttle vector that contains the complete 2 $\mu$  sequence, as described in Broach, *Molecular Biology of the Yeast Saccharomyces*, Cold Spring Harbor Press 1:445 (1981) and pBR322 sequences. It also contains

the yeast URA3 gene derived from plasmid YEp24, as described in Botstein, et al., *Gene* 8:17 (1979) and the yeast LEU<sup>2d</sup> gene derived from plasmid pC1/1, as described in European Patent Application, Publication No. 116 201. Plasmid pAB24 was constructed by digesting YEp24 with EcoRI and religating the vector to remove the partial 2μ sequences. The resulting plasmid, YEp24ΔR1, was linearized by digestion with ClaI and ligated with the complete 2μ plasmid which had been linearized with ClaI. The resulting plasmid, pCBou, was then digested with XbaI and the 8605 bp vector fragment was gel isolated. This isolated XbaI fragment was ligated with a 4460 bp XbaI fragment containing the Leu<sup>2d</sup> gene isolated from pC1/1; the orientation of the LEU<sup>2d</sup> gene is in the same direction as the URA3 gene. Insertion of the expression cassette was in the unique BamHI site of the pBR322 sequences, this interrupting the gene for bacterial resistance to tetracycline.

Plasmid pBS24 is a derivative of pAB24 as described. Plasmid pAB24 was digested with BamHI and SalI, which cut within the tetracycline gene of the pBR322 sequences, and gel purified. The vector was then ligated with a synthetic adapter of the following sequence which created new unique BglII and BamHI sites:

(SEQ ID NO: 15)  
 BglII BamHI  
 5' GATCAGATCTAAATTTCCTGGATCC-3'  
 TCTAGATTTAAAGGCGCTAGGAGCT (SEQ ID NO: 16)  
 (BamHI) (SalI)

The resulting vector, pAB24ΔBL was then digested with BamHI and BglII and gel purified. The linearized vector was ligated with the BamHI cassette excised and purified from pSOD/env-5b to give pBS24. The cassette contains the hybrid ADH-2/GAPDH promoter and α-factor terminator with an NcoI-SalI insert of the SOD/env-5b fusion gene. The cassette is oriented in pBS24 such that the direction of transcription from the ADH-2/GAPDH promoter is in the opposite direction to that of the inactivated tetracycline gene of the pBR322 sequences.

pBS24.1 contains a BamHI/SalI fragment encoding the human FGF protein instead of the SOD/env-5b in pBS24, as described in U.S. Pat. No. 5,156,949. The relevant vector

SEQ ID NO: 18 (sense)

Met Ser Tyr Asp Tyr Met Glu Gly Gly\*  
 5' GT TGT TTC ATG AGT TAT GAT TAC ATG GAA OGA GGG  
 BspHI

SEQ ID NO: 14 (antisense)

Stop Thr Ile Ala Met Pro Leu Phe His\*\*  
 5' GGT GGT GTC GAC TTA AGT TAT TGC CAT AGG AAG AAA GTG 3'  
 SalI

\*The peptide sequence is listed as SEQ ID NO: 17.

\*\*As encoded by the reverse complement of the primer, and the peptide sequence is listed as SEQ ID NO: 12.

fragment of pBS24.1 is present in pBS24.1bBMP. pBS24.1bBMP is the same as pBS24.1 except it contains a BamHI/SalI bovine BMP expression cassette instead of the human FGF protein. The vector pBS24.1bBMP was deposited with the ATCC, Rockville, Md., U.S.A., on 1 Jun. 1989 under Accession no. 20949.

#### Example 7

##### Expression of rKGF<sub>der1-23</sub> by Yeast Cells

The procedure for expression of rKGF<sub>der1-23</sub> in accordance with the present invention:

The secretory expression vector: as described in Example 6.

Host for expression: *Saccharomyces cerevisiae*.

Method of introducing the expression vector into host cells: by electroporation, as described in "Guide to Yeast Genetics & Molecular Biology," in *Methods in Enzymology*, Vol. 194 (Academic Press 1991).

Selection of transformants: on ura with 2% glucose medium.

Seed culture of transformants: overnight incubation in 1 ml of leu with 2% glucose medium at 30° C. in a shaking apparatus.

For production of rKGF<sub>der1-23</sub> expression: 20 ml culture seeded with the overnight culture in ura with 2% glucose medium, for approximately 72 hours.

#### Example 8

##### Construction of a KGF<sub>der1-23</sub> Yeast Vector for Intracellular Yeast Expression

This example describes a procedure for construction of a yeast expression vector, for intracellular expression of the KGF<sub>der1-23</sub> by transformed yeast cells, in accordance with the present invention:

Specifically, the components of the expression vector: KGF<sub>der1-23</sub> coding sequence, a hybrid yeast promoter and an α-factor terminator.

The fragments for construction of this vector:

- (1) BamHI/NcoI fragment containing the ADH2/GAPDH promoter;
- (2) BspHI/SalI fragment encoding KGF<sub>der1-23</sub>; and
- (3) BamHI/SalI vector fragment of the yeast expression vector, pBS24.1, containing the *Saccharomyces cerevisiae* α-factor terminator, leucine and uracil yeast selectable markers, and 2μ sequences as an origin of replication.

##### A. Construction of a KGF<sub>der1-23</sub> Coding Sequence

Construction of the BspHI/SalI KGF<sub>der1-23</sub> coding sequence fragment: by PCR using the pAcc/KGF baculovirus transfer vector, as described in Example 1 and 2.

The primers for PCR:

Reagents for construction of the BspHI SalI KGF<sub>der1-23</sub> coding sequence by PCR: 1 μl of each primer (100 pmole/μl), 1 μl of the template, pGAI7, at ~500 μg/ml, and the following reagents from Perkin Elmer PCR kit (Norwalk, Conn.): 8 μl of dNTP (1.25 mM each of dATP, dTTP, dCTP, and dGTP), 10 μl of 10x buffer, 0.5 μl of Taq polymerase, and 63.5 μl of water. The temperature cycle for PCR: 94° C. for 1 minute, 37° C. for 2 minutes, and 72° C. for 3 minutes. Number of repetitions of the temperature cycle: 30. Purification of the PCR product of ~414 bp by gel purification, and by use of a GeneClean kit by bio101, La Jolla, Calif., U.S.A.

## B. Construction of a BamHI/NcoI Promoter Fragment

The source of the BamHI/NcoI promoter fragment: by digestion of pSI3 containing a superoxidase dismutase (SOD)/insulin fusion yeast expression cassette comprising a BamHI/NcoI ADH2/GAPDH hybrid promoter, to release a fragment of about 1366 bp containing the hybrid promoter sequences.

pSI3 is a derivative of pYASII, which was deposited with the ATCC, Rockville, Md., U.S.A. on 27 Feb. 1985, and assigned Accession no. 20745. The construction of pYASII is described in U.S. Pat. No. 4,751,180. pYASII contains the same BamHI/NcoI hybrid promoter fragment as pSI3.

## C. Construction of the BamHI/SalI pBS24.1 Vector Fragment

Source of BamHI/SalI pBS24.1 vector fragment: by digestion of pBS24.1 with BamHI and SalI and isolation of the large vector fragment, as described in Example 6, section C.

## Example 9

Intracellular Expression of rKGF<sub>der1-23</sub> by Yeast Cells

Procedure for intracellular expression of rKGF<sub>der1-23</sub> by yeast cells in accordance with the present invention:

Expression vector for intracellular expression in yeast cells: as described in Example 8.

Host cells for transformation: *Saccharomyces cerevisiae*.

Method of introducing expression vector into host cells: by electroporation, was described in "Guide to Yeast Genetics & Molecular Biology," *Methods in Enzymology*, Vol. 194 (Academic Press, 1991).

Selection of transformants: on ura with 2% glucose medium.

Seed culture of transformants: overnight incubation in 1 ml of leu with 2% glucose medium at 30° C. in a shaking apparatus. Culture for production of rKGF<sub>der1-23</sub>: a 20 ml

culture seeded with the overnight culture in ura with 2% glucose medium for approximately 72 hours.

## Deposit Information:

The following materials were deposited with the American Type Culture Collection:

Virus Name	Deposit Date	Accession No.
<i>Autographa californica</i> nuclearpolyhedrosis virus KGF-5 VR2411	17 Jun 1993	
<i>Escherichia coli</i> , pYGA17	29 Dec 1997	67597
<i>Saccharomyces cerevisiae</i> pBS24.1bBMP	1 Jun 1989	20949
<i>Saccharomyces cerevisiae</i> , 2150-2-3 pYASII	27 Feb 1985	20745

The above materials have been deposited with the American Type Culture Collection, Rockville, Md., under the accession numbers indicated. This deposit will be maintained under the terms of the Budapest Treaty on the International Recognition of the Deposit of Microorganisms for purposes of Patent Procedure. The deposits will be maintained for a period of 30 years following issuance of this patent, or for the enforceable life of the patent, whichever is greater. Upon issuance of the patent, the deposits will be available to the public from the ATCC without restriction.

These deposits are provided merely as convenience to those of skill in the art, and are not an admission that a deposit is required under 35 U.S.C. §112. The sequence of the polynucleotides contained within the deposited materials, as well as the amino acid sequence of the polypeptides encoded thereby, are incorporated herein by reference and are controlling in the event of any conflict with the written description of sequences herein. A license may be required to make, use, or sell the deposited materials, and no such license is granted hereby.

## SEQUENCE LISTING

## (1) GENERAL INFORMATION:

(iii) NUMBER OF SEQUENCES: 19

## (2) INFORMATION FOR SEQ ID NO:1:

## (i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 194 amino acids
- (B) TYPE: amino acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

## (ii) MOLECULE TYPE: protein

## (xi) SEQUENCE DESCRIPTION: SEQ ID NO:1:

```

Met His Lys Trp Ile Leu Thr Trp Ile Leu Pro Thr Leu Leu Tyr Arg
1          5          10          15
Ser Cys Phe His Ile Ile Cys Leu Val Gly Thr Ile Ser Leu Ala Cys
20          25          30
Asn Asp Met Thr Pro Glu Glu Met Ala Thr Asn Val Asn Cys Ser Ser
35          40          45
Pro Glu Arg His Thr Arg Ser Tyr Asp Tyr Met Glu Gly Gly Asp Ile
50          55          60
Arg Val Arg Arg Leu Phe Cys Arg Thr Glu Trp Tyr Leu Arg Ile Asp

```

-continued

65		70		75		80									
Lys	Arg	Gly	Lys	Val	Lys	Gly	Thr	Gln	Glu	Met	Lys	Asn	Asn	Tyr	Asn
				85					90					95	
Ile	Met	Glu	Ile	Arg	Thr	Val	Ala	Val	Gly	Ile	Val	Ala	Ile	Lys	Gly
			100					105					110		
Val	Glu	Ser	Glu	Phe	Tyr	Leu	Ala	Met	Asn	Lys	Glu	Gly	Lys	Leu	Tyr
		115					120					125			
Ala	Lys	Lys	Glu	Cys	Asn	Glu	Asp	Cys	Asn	Phe	Lys	Glu	Leu	Ile	Leu
		130				135					140				
Glu	Asn	His	Tyr	Asn	Thr	Tyr	Ala	Ser	Ala	Lys	Trp	Thr	His	Asn	Gly
		145			150					155					160
Gly	Glu	Met	Phe	Val	Ala	Leu	Asn	Gln	Lys	Gly	Ile	Pro	Val	Arg	Gly
			165						170					175	
Lys	Lys	Thr	Lys	Lys	Glu	Gln	Lys	Thr	Ala	His	Phe	Leu	Pro	Met	Ala
			180				185						190		
Ile	Thr														

## ( 2 ) INFORMATION FOR SEQ ID NO:2:

## ( i ) SEQUENCE CHARACTERISTICS:

- ( A ) LENGTH: 23 amino acids
- ( B ) TYPE: amino acid
- ( C ) STRANDEDNESS: single
- ( D ) TOPOLOGY: linear

## ( ii ) MOLECULE TYPE: protein

## ( xi ) SEQUENCE DESCRIPTION: SEQ ID NO:2:

Cys	Asn	Asp	Met	Thr	Pro	Glu	Gln	Met	Ala	Thr	Asn	Val	Asn	Cys	Ser
1				5					10					15	
Ser	Pro	Glu	Arg	His	Thr	Arg									
			20												

## ( 2 ) INFORMATION FOR SEQ ID NO:3:

## ( i ) SEQUENCE CHARACTERISTICS:

- ( A ) LENGTH: 6 amino acids
- ( B ) TYPE: amino acid
- ( C ) STRANDEDNESS: single
- ( D ) TOPOLOGY: linear

## ( ii ) MOLECULE TYPE: peptide

## ( xi ) SEQUENCE DESCRIPTION: SEQ ID NO:3:

Met	His	Lys	Trp	Ile	Leu
1				5	

## ( 2 ) INFORMATION FOR SEQ ID NO:4:

## ( i ) SEQUENCE CHARACTERISTICS:

- ( A ) LENGTH: 35 base pairs
- ( B ) TYPE: nucleic acid
- ( C ) STRANDEDNESS: single
- ( D ) TOPOLOGY: linear

## ( ii ) MOLECULE TYPE: DNA (genomic)

## ( xi ) SEQUENCE DESCRIPTION: SEQ ID NO:4:

AGATCTCTGC AGCTATAATG CACAAATGGA TACTG

35

## ( 2 ) INFORMATION FOR SEQ ID NO:5:

## ( i ) SEQUENCE CHARACTERISTICS:

- ( A ) LENGTH: 7 amino acids

-continued

- ( B ) TYPE: amino acid
- ( C ) STRANDEDNESS: single
- ( D ) TOPOLOGY: linear

( i i ) MOLECULE TYPE: peptide

( x i ) SEQUENCE DESCRIPTION: SEQ ID NO:5:

Thr Ile Ala Met Pro Leu Phe  
1 5

( 2 ) INFORMATION FOR SEQ ID NO:6:

- ( i ) SEQUENCE CHARACTERISTICS:
  - ( A ) LENGTH: 38 base pairs
  - ( B ) TYPE: nucleic acid
  - ( C ) STRANDEDNESS: single
  - ( D ) TOPOLOGY: linear

( i i ) MOLECULE TYPE: DNA (genomic)

( x i ) SEQUENCE DESCRIPTION: SEQ ID NO:6:

AGATCTGCGG CCGCTTAAGT TATTOCCATA GGAAGAAA

38

( 2 ) INFORMATION FOR SEQ ID NO:7:

- ( i ) SEQUENCE CHARACTERISTICS:
  - ( A ) LENGTH: 20 amino acids
  - ( B ) TYPE: amino acid
  - ( C ) STRANDEDNESS: single
  - ( D ) TOPOLOGY: linear

( i i ) MOLECULE TYPE: peptide

( x i ) SEQUENCE DESCRIPTION: SEQ ID NO:7:

Ser Tyr Asp Tyr Met Glu Gly Gly Asp Ile Arg Val Arg Arg Leu Phe  
1 5 10 15  
Xaa Arg Thr Gln  
20

( 2 ) INFORMATION FOR SEQ ID NO:8:

- ( i ) SEQUENCE CHARACTERISTICS:
  - ( A ) LENGTH: 32 base pairs
  - ( B ) TYPE: nucleic acid
  - ( C ) STRANDEDNESS: single
  - ( D ) TOPOLOGY: linear

( i i ) MOLECULE TYPE: DNA (genomic)

( x i ) SEQUENCE DESCRIPTION: SEQ ID NO:8:

GGTGGTGGAT CCCCAGCTTA GTTCATAAGT CC

32

( 2 ) INFORMATION FOR SEQ ID NO:9:

- ( i ) SEQUENCE CHARACTERISTICS:
  - ( A ) LENGTH: 12 amino acids
  - ( B ) TYPE: amino acid
  - ( C ) STRANDEDNESS: single
  - ( D ) TOPOLOGY: linear

( i i ) MOLECULE TYPE: peptide

( x i ) SEQUENCE DESCRIPTION: SEQ ID NO:9:

His Gln Asn Val Phe Arg Lys Ala Pro Ile Gln Ala  
1 5 10

( 2 ) INFORMATION FOR SEQ ID NO:10:

- ( i ) SEQUENCE CHARACTERISTICS:

-continued

( A ) LENGTH: 36 base pairs  
 ( B ) TYPE: nucleic acid  
 ( C ) STRANDEDNESS: single  
 ( D ) TOPOLOGY: linear

( i i ) MOLECULE TYPE: DNA (genomic)

( x i ) SEQUENCE DESCRIPTION: SEQ ID NO:10:

GTGTTGGTTA ACGAATCGCT TAGCCGGAAT TTGTGC

36

( 2 ) INFORMATION FOR SEQ ID NO:11:

( i ) SEQUENCE CHARACTERISTICS:  
 ( A ) LENGTH: 12 amino acids  
 ( B ) TYPE: amino acid  
 ( C ) STRANDEDNESS: single  
 ( D ) TOPOLOGY: linear

( i i ) MOLECULE TYPE: peptide

( x i ) SEQUENCE DESCRIPTION: SEQ ID NO:11:

Pro Ala Lys Arg Ser Tyr Asp Tyr Met Glu Gly Gly  
 1 5 10

( 2 ) INFORMATION FOR SEQ ID NO:12:

( i ) SEQUENCE CHARACTERISTICS:  
 ( A ) LENGTH: 39 base pairs  
 ( B ) TYPE: nucleic acid  
 ( C ) STRANDEDNESS: single  
 ( D ) TOPOLOGY: linear

( i i ) MOLECULE TYPE: DNA (genomic)

( x i ) SEQUENCE DESCRIPTION: SEQ ID NO:12:

CCGCCGGCTA AGCGAAATTA TGATTACATG GAAGGAGGG

39

( 2 ) INFORMATION FOR SEQ ID NO:13:

( i ) SEQUENCE CHARACTERISTICS:  
 ( A ) LENGTH: 8 amino acids  
 ( B ) TYPE: amino acid  
 ( C ) STRANDEDNESS: single  
 ( D ) TOPOLOGY: linear

( i i ) MOLECULE TYPE: peptide

( x i ) SEQUENCE DESCRIPTION: SEQ ID NO:13:

Thr Ile Ala Met Pro Leu Phe His  
 1 5

( 2 ) INFORMATION FOR SEQ ID NO:14:

( i ) SEQUENCE CHARACTERISTICS:  
 ( A ) LENGTH: 39 base pairs  
 ( B ) TYPE: nucleic acid  
 ( C ) STRANDEDNESS: single  
 ( D ) TOPOLOGY: linear

( i i ) MOLECULE TYPE: DNA (genomic)

( x i ) SEQUENCE DESCRIPTION: SEQ ID NO:14:

GGTGGTGTCTG ACTTAAATTA TTGCCATAGG AAGAAAATG

39

( 2 ) INFORMATION FOR SEQ ID NO:15:

( i ) SEQUENCE CHARACTERISTICS:  
 ( A ) LENGTH: 25 base pairs  
 ( B ) TYPE: nucleic acid  
 ( C ) STRANDEDNESS: single



-continued

( D ) TOPOLOGY: linear

( i i ) MOLECULE TYPE: DNA (genomic)

( x i ) SEQUENCE DESCRIPTION: SEQ ID NO:15:

GATCAGATCT AAATTTCCCG GATCC

25

( 2 ) INFORMATION FOR SEQ ID NO:16:

( i ) SEQUENCE CHARACTERISTICS:

( A ) LENGTH: 25 base pairs

( B ) TYPE: nucleic acid

( C ) STRANDEDNESS: single

( D ) TOPOLOGY: linear

( i i ) MOLECULE TYPE: DNA (genomic)

( x i ) SEQUENCE DESCRIPTION: SEQ ID NO:16:

TCTAGATTTA AAGGGCCTAG GAGCT

25

( 2 ) INFORMATION FOR SEQ ID NO:17:

( i ) SEQUENCE CHARACTERISTICS:

( A ) LENGTH: 9 amino acids

( B ) TYPE: amino acid

( C ) STRANDEDNESS: single

( D ) TOPOLOGY: linear

( i i ) MOLECULE TYPE: DNA (genomic)

( x i ) SEQUENCE DESCRIPTION: SEQ ID NO:17:

Met Ser Tyr Asp Tyr Met Glu Gly Gly  
1 5

( 2 ) INFORMATION FOR SEQ ID NO:18:

( i ) SEQUENCE CHARACTERISTICS:

( A ) LENGTH: 35 base pairs

( B ) TYPE: nucleic acid

( C ) STRANDEDNESS: single

( D ) TOPOLOGY: linear

( i i ) MOLECULE TYPE: DNA (genomic)

( x i ) SEQUENCE DESCRIPTION: SEQ ID NO:18:

GTTGTTTCAT GAGTTATGAT TACATGGAAG GAGGG

35

( 2 ) INFORMATION FOR SEQ ID NO:19:

( i ) SEQUENCE CHARACTERISTICS:

( A ) LENGTH: 99 base pairs

( B ) TYPE: nucleic acid

( C ) STRANDEDNESS: single

( D ) TOPOLOGY: linear

( i i ) MOLECULE TYPE: DNA (genomic)

( i x ) FEATURE:

( A ) NAME/KEY: -

( B ) LOCATION: 13..14

( D ) OTHER INFORMATION: /note= "The figure did not contain  
the intervening polyhedrin sequences."

( x i ) SEQUENCE DESCRIPTION: SEQ ID NO:19:

TATAAATATT CCGGGCGCGG ATCGGTACCA GATCTOCAGA ATTCTAGAGG ATCCTGATCA 60

GCTAOCAGAG CTCGCGGCGG CCCGGGCCGT ACCGACTCT

99

What is claimed:

1. A keratinocyte growth factor fragment that exhibits at least a 2-fold increase in mitogenic activity as compared to a mature, recombinant, full-length keratinocyte growth factor, wherein the fragment lacks the first 23 N-terminal amino acid residues of the mature, full-length keratinocyte growth factor but retains the remainder of the molecule.

2. The keratinocyte growth factor fragment as claimed in claim 1, wherein the fragment exhibits a 7-fold increase in mitogenic activity as compared to the mature, recombinant, full-length keratinocyte growth factor.

3. The keratinocyte growth factor fragment as claimed in claim 1, wherein the fragment exhibits a 10-fold increase in mitogenic activity as compared to the mature, recombinant, full-length keratinocyte growth factor.

4. The keratinocyte growth factor fragment as claimed in claim 1, wherein the fragment exhibits decreased cytotoxicity as compared to the mature, recombinant, full-length keratinocyte growth factor.

5. A composition comprising:

(a) a keratinocyte growth factor fragment that exhibits at least a 2-fold increase in mitogenic activity as compared to the mature, recombinant, full-length keratinocyte growth factor, wherein the fragment lacks the first 23 N-terminal amino acid residues of the mature, full-length keratinocyte growth factor but retains the remainder of the molecule, and

(b) a carrier.

6. The keratinocyte growth factor fragment as claimed in claim 1 having the amino acid sequence depicted at amino acid residues 24 to 163, inclusive, of FIG. 1 (amino acid residues 55-194, inclusive, of SEQ ID No: 1).

7. An analog of the keratinocyte growth factor fragment as claimed in claim 6, wherein the analog differs from its

keratinocyte growth factor fragment of claim 6 in having at least one conservative substitution selected from the group of substitutions consisting of a substitution of a leucine with an isoleucine or an isoleucine with a leucine, a substitution of a leucine with a valine or a valine with a leucine, a substitution of an aspartic acid with a glutamic acid or a glutamic acid with an aspartic acid and a substitution of a threonine with a serine or a serine with a threonine and further wherein the analog exhibits the biological activity of the keratinocyte growth factor fragment.

8. An analog of the keratinocyte growth factor fragment as claimed in claim 6, wherein the analog differs from its keratinocyte growth factor fragment of claim 6 in having at least one cysteine residue substituted by another amino acid and further wherein the analog exhibits the biological activity of the keratinocyte growth factor fragment.

9. The analog as claimed in claim 8, wherein the cysteine residue is replaced with serine or threonine.

10. A composition comprising:

(a) the keratinocyte growth factor fragment as claimed in claim 6, and

(b) a carrier.

11. A composition comprising:

(a) the analog as claimed in claim 7, and

(b) a carrier.

12. A composition comprising:

(a) the analog as claimed in claim 5, and

(b) a carrier.

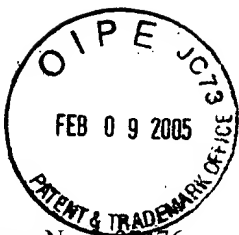
13. A composition comprising:

(a) the analog as claimed in claim 9, and

(b) a carrier.

\* \* \* \* \*

## Exhibit B



Customer Num: 27476



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Emeryville CA 94662-8097

## **MAINTENANCE FEE STATEMENT**

The data shown below is from the records of the Patent and Trademark Office. If the maintenance fees and any necessary surcharges have been timely paid for the patents listed below, the notation "PAID" will appear in column 10, "STAT", below.

If a maintenance fee payment is defective, the reason is indicated by code in column 10, "STAT" below. TIMELY CORRECTION IS REQUIRED IN ORDER TO AVOID EXPIRATION OF THE PATENT. NOTE 37 CFR 1.377. THE PAYMENT(S) WILL BE ENTERED UPON RECEIPT OF ACCEPTABLE CORRECTION. IF PAYMENT OR CORRECTION IS SUBMITTED DURING THE GRACE PERIOD, A SURCHARGE IS ALSO REQUIRED. NOTE 37 CFR 1.20(k) and (l).

If the statement of small entity status is defective the reason is indicated below in column 10 for the related patent number. THE STATEMENT OF SMALL ENTITY STATUS WILL BE ENTERED UPON RECEIPT OF ACCEPTABLE CORRECTION.

PATENT NUMBER	FEE CODE	FEE AMT	SUR CHARGE	APPLICATION NUMBER	PATENT DATE	FILE DATE	PAY YR	SML ENT	STAT	ATTY DKT NUM
5,677,278	183	\$850.00	\$0.00	08/410,941	10/14/97	03/27/95	04	NO	PAID	0953.002

**DIRECT YOUR RESPONSE TOGETHER WITH ANY QUESTIONS ABOUT THIS NOTICE TO:**  
**Mail Stop: M. Correspondence, Director of the United States Patent and Trademark Office**  
**P.O. Box 1450**  
**Alexandria, VA 22313-1450**

## Exhibit C



Atty Dkt No. PP0953.002  
2300-0953.01  
PATENT

I hereby certify that this correspondence is being deposited with the United States Postal Service as first class mail in an envelope addressed to: Commissioner for Patents, P. O. Box 1450, Alexandria, VA 22313-1450 on:

1/11/05  
Date

  
Signature

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In Re Letters Patent of:

GOSPODAROWICZ et al.

Serial No.: 08/410,941

Filing Date: March 27, 1995

Patent No.: 5,677,278

Grant Date: October 14, 1997

Title: TRUNCATED KERATINOCYTE GROWTH FACTOR (KGF) HAVING  
INCREASED BIOLOGICAL ACTIVITY

TRANSMITTAL LETTER

Commissioner for Patents  
P. O. Box 1450  
Alexandria, VA 22313-1450

Sir:

Please furnish the undersigned with a Certificate of Correction for the above-identified patent. The correction is as follows:

In The Claims

12. A composition comprising:

- (a) the analog as claimed in claim 5 8, and
- (b) a carrier.

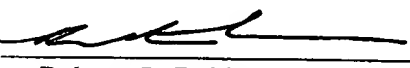
Remarks

A Certificate of Correction is requested in order to correct a PTO error. Issued claim 12 depends from claim 8, not claim 5. Please see amendment filed on January 26, 1995. Claim 32 of the amendment filed on January 26, 1995 corresponds to issued claim 12.

A Certificate of Correction in duplicate is enclosed. No fee is believed due for this paper, however, if any fee is found to be necessary the Commissioner is authorized to charge our Deposit Account No. 18-1648 therefor.

Respectfully submitted,

Date: 1/11/05

By:   
Roberta L. Robins  
Registration No. 33,208

Gerald Suh  
Chiron Corporation  
Intellectual Property - R440  
P.O. Box 8097  
Emeryville, CA 94662-8097  
Telephone: (510) 923-3888  
Facsimile: (510) 655-3542.

UNITED STATES PATENT AND TRADEMARK OFFICE

**CERTIFICATE OF CORRECTION**

PATENT NO. : 5,677,278

DATED : October 14, 1997

INVENTOR(S) : Denis J. Gospodarowicz and Frank R. Masiarz

It is certified that error appears in the above-identified patent and that said Letters Patent is hereby corrected as shown below:

In The Claims

12. A composition comprising:

- (a) the analog as claimed in claim 5 8, and
- (b) a carrier.

MAILING ADDRESS OF SENDER

ROBINS & PASTERNAK LLP  
1731 Embarcadero Road, Suite 230  
Palo Alto, CA 94303

PATENT NO. 5,677,278

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